Defining the role of NCR- ILC3s in chronic colitis

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Defining the role of NCR$^-$ ILC3s in chronic colitis

A thesis submitted to the School of Immunology & Microbial Sciences at King's College London for the degree of Doctor of Philosophy

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To Randolph J Noelle

For showing me the way and raising the bar so high…

Abstract

Innate lymphoid cells (ILCs) are relatively newly identified cells that have a lymphoid-like morphology, but lack antigen specific receptors. Similarly to T cells, they can be divided into three main groups (1, 2 and 3), which produce distinct combinations of cytokines to mediate their functions, and whose development depends on different transcriptional factors. Group 3 ILCs (ILC3s) are enriched at mucosal surfaces, where they act as key effector cells. They can be further subdivided into lymphoid tissue inducer (LTi) cells, and NCR^+ or NCR^- ILC3s based on whether or not they express natural cytotoxicity receptors. NCR^- ILC3s, which co-produce IL-22 and IL-17A, remain poorly understood, especially with regards to their function in the colon.

In this thesis it was shown that NCR^- ILC3s were the dominant ILC3 subset in the healthy colon, as well as during colonic inflammation, and that NCR^- ILC3s drived colitis in *Tbx21*$^{-/-}$ *x Rag2*$^{-/-}$ Ulcerative Colitis (TRUC) mice with IL-22 being their effector cytokine. NCR^- ILC3 derived IL-22 triggered endoplasmic reticulum (ER) stress in colonic epithelial cells and induced CXCL1 and CXCL5 secretion that subsequently led to neutrophil recruitment to the inflamed colon. These pro-inflammatory actions of IL-22 were augmented by IL-17A, another cytokine produced by NCR^- ILC3s that is strongly implicated in inflammatory bowel disease (IBD) pathogenesis. Whole transcriptome analysis of colonic biopsies from patients with Ulcerative colitis (UC) and healthy individuals revealed compelling evidence that these pro-inflammatory, IL-22 dependent pathways may be relevant in human disease.

Taken all together these data shed more light into TRUC disease, and possibly provides new insights into the immunopathology of chronic colitis. Targeting the pro-inflammatory pathways mediated by NCR^- ILC3s may represent a novel therapeutic approach for IBD.

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Abbreviations

4-PBA	4-phenylbutyric acid
ACK	Ammonium-Chloride-Potassium (lysis buffer)
AHR	Aryl hydrocarbon receptor
AMP	Antimicrobial peptide
APC	Antigen presenting cell
ATG16L1	Autophagy-related 16 like 1 protein
BRC	Biomedical research council
CD	Cluster of differentiation or Crohn's disease
CLP	Common lymphoid progenitor
DAI	Disease activity index
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DNBS	Dinitrobenzene sulfonic acid
DSS	Dextran sodium sulphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum

FC	Flow cytometry
FCS	Fetal calf serum
GATA-3	GATA binding protein 3
GC	Glucocorticoid
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSEA	Gene set enrichment analysis
GWAS	Genome wide association studies
H&E	Haematoxylin and eosin
HT	*Helicobacter Typhlonius*
IBD	Inflammatory bowel disease
IFNγ	Interferon γ
IL	Interleukin
ILC	Innate lymphoid cell
ip.	Intraperitoneally
ir.	Intrarectally
KCL	King's College London
KI	Knock in
KLRG1	Killer cell lectin-like receptor subfamily G member 1
KO	Knock out
Lin$^-$	Lineage negative (-)
LP	Lamina propria

LTi	Lymphoid tissue inducer
mAb	Monoclonal antibody
MAP	Mitogen-activated protein
MDP	Muramyl dipeptide
MFI	Mean fluorescence intensity
MHC II	Major histocompatibility complex (class) II
MLN	Mesenteric lymph node
MP	Mononuclear phagocyte
mRNA	Messenger ribonucleic acid
Mφ	Macrophage
NF- ϰB	Nuclear factor ϰB
NK	Natural killer
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
o/n	Overnight
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PLZF	Promyeloid leukaemia zinc finger
PMA	Phorbol 12-myristate 13-acetate
QMUL	Queen Mary University of London
Rag1	Recombination activating gene 1

Rag2	Recombination activating gene 2
RORγt	Retinoic acid related orphan receptor γt
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
RT-qPCR	Real time quantitative polymerase chain reaction
SCID	Severe combined immune deficiency
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
SPF	Specific pathogen free
STAT	Signal transducer and activator of transcription
T-bet	T-box transcription factor
Tbx21	T-box transcription factor 21
TCT	T cell transfer
Th	T helper
TL1A	TNF-like protein 1A
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNFSF15	Tumour necrosis factor (ligand) superfamily member 15
TNFα	Tumour necrosis factor α
Treg	Regulatory T cell
TRUC	*Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ Ulcerative Colitis
TRUC*Il22*$^{-/-}$	*Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ Ulcerative Colitis x *Il22*$^{-/-}$

TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis
UPR	Unfolded protein response
WT	Wild type
αLP	α lymphoid precursor

Publications related to/arising from this thesis

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Chapter 1

Introduction

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a medical term used to describe chronic intestinal inflammation. IBD can be divided into two main distinct conditions: *Crohn's disease* (CD) where inflammation can affect any part of the gastrointestinal tract, and *Ulcerative colitis* (UC) where inflammation is limited to the colon (Baumgart, Sandborn 2007). Both forms are characterized by alternating phases of clinical relapse and remission, and are commonly presented with symptoms such as diarrhoea, rectal bleeding, mucus discharge and abdominal pain (Baumgart, Sandborn 2007). In some cases, inflammation can also extend to organs beyond the digestive system, including the eyes (Mintz, Feller et al. 2004), joints (Orchard, Wordsworth et al. 1998), skin (Lebwohl, Lebwohl 1998) and liver (Raj, Lichtenstein 1999). IBD patients are at greater risk of developing another chronic inflammatory disorder such as ankylosing spondylitis, psoriasis (Loftus 2004) and colorectal cancer (Eaden, Abrams et al. 2001, Rutter, Saunders et al. 2006). Despite the severity of these symptoms that undoubtedly have a negative impact on psychology (Graff, Walker et al. 2009, Trachter, Rogers et al. 2002) and quality of life (Jelsness-Jorgensen, Bernklev et al. 2011, Bernstein, Kraut et al. 2001) many IBD patients may have to undergo disfiguring surgery (Baumgart, Sandborn 2007). Although IBD incidence is generally thought to spike in the western world countries (more than 3 million people affected in Europe and North America), it is now progressively rising in the developing world (Loftus 2004, Lakatos 2006).

The etiology of IBD is complex and remains poorly understood. Familial studies show that first-degree relatives have higher risk of developing IBD than the rest of the population (Tysk, Lindberg et al. 1988, Orholm, Munkholm et al. 1991). Furthermore, monozygotic twins are more likely to be affected than dizygotic twins, especially in CD (Orholm, Munkholm et al. 1991) suggesting genetic predisposition. In a landmark study done by the Wellcome Trust Case Control Consortium in 2007,

CD was found to have the highest relative-sibling risk among the 7 diseases studied (Wellcome Trust Case Control Consortium 2007). More recent data show single nucleotide polymorphisms (SNPs) shared by CD and UC, while other variants are unique for CD or UC (Fisher, Tremelling et al. 2008, Franke, Balschun et al. 2008a). In spite of the compelling evidence that IBD is a genetic-associated disease, genetic factors are insufficient to be the sole cause, as concordance between monozygotic twins is generally less than 50% (Halfvarson, Bodin et al. 2003, Jess, Riis et al. 2005, Spehlmann, Begun et al. 2008). Environmental factors such as diet (Hansen, Jess et al. 2011, Jakobsen, Paerregaard et al. 2013, Turner, Zlotkin et al. 2009), smoking (Sutherland, Ramcharan et al. 1990), or exposure to gastrointestinal infections (Hansen, Jess et al. 2011, Garcia Rodriguez, Ruigomez et al. 2006, Gradel, Nielsen et al. 2009, Porter, Tribble et al. 2008) may also contribute to IBD pathogenesis. Moreover, many studies show a role for the microbiome, with antibiotics used to prevent recurrence in CD patients undergoing surgery, or to treat pouchitis in UC patients following colectomy (Rutgeerts, Hiele et al. 1995, Shen, Achkar et al. 2001). Overall, it appears that both genetic and environmental factors have an impact on the development of IBD.

1.1.1 Genetic contributions to IBD pathogenesis

In the last decade, great progress has been made in unravelling genetic factors that are associated with altered IBD risk, or even contribute to IBD pathogenesis. Genome-wide association studies (GWAS) lead the field by providing unbiased correlations between gene variations and disease occurrence. More than one hundred and sixty genetic loci are associated with IBD susceptibility so far, including genes involved in bacterial recognition, epithelial barrier integrity and immune activation (Jostins, Ripke et al. 2012). Some genes are shared between the two forms of IBD, while others are only associated with either CD or UC susceptibility (Jostins, Ripke et al. 2012). Genetic loci associated with IBD risk also overlap with susceptibility to other immune mediated diseases such as primary sclerosing cholangitis, ankylosing spondylitis and psoriasis (Bernstein, Wajda et al. 2005), as well as immunodeficiencies and infections (Jostins, Ripke et al. 2012).

The first ever described polymorphisms associated with risk for Crohn's disease were found in the nucleotide-binding oligomerization domain-containing protein 2

(NOD2) locus (Hugot, Chamaillard et al. 2001, Ogura, Bonen et al. 2001). NOD2 gene encodes for an intracellular pattern recognition receptor that senses bacterial peptidoglycans and can be activated by a small peptidoglycan component known as muramyl dipeptide (MDP) resulting in the activation of nuclear factor κB (NF-κB) and mitogen-activated protein (MAP) kinase signalling pathways (Girardin, Travassos et al. 2003, Inohara, Ogura et al. 2003, Kobayashi, Chamaillard et al. 2005). Each one of the three SNPs that are located near or within the leukine-rich repeat region of the NOD2 protein (the domain needed for bacterial sensing) are highly associated with CD susceptibility (Abraham, Cho 2006). Interestingly, they are found in European descent individuals, but not in Japanese, Chinese and Korean affected individuals (Croucher, Mascheretti et al. 2003, Leong, Armuzzi et al. 2003, Yamazaki, Takazoe et al. 2002), while they are rare in African American IBD patients (Kugathasan, Loizides et al. 2005). Disease penetrance is yet limited even for homozygous individuals (Hugot, Zaccaria et al. 2007) suggesting that NOD2 mutations alone are insufficient to induce CD, which is also corroborated by the fact that NOD2 KO mice do not develop intestinal inflammation (Kobayashi, Chamaillard et al. 2005, Pauleau, Murray 2003).

In addition to NOD2 gene, GWAS have identified many more genetic loci of the innate immune system to be associated with IBD risk. Great examples include genes involved in autophagy, a cellular process important for degradation of intracellular pathogens, antigen processing and cell signalling regulation. A SNP in the ATG16L1 gene that leads to the conversion of the amino acid alanine to threonine (Ala281Thr) is found to be highly associated with CD susceptibility (Hampe, Franke et al. 2007, Prescott, Fisher et al. 2007, Rioux, Xavier et al. 2007). CD patients with the ATG16L1 SNP have abnormal Paneth cell morphology, as do mice with low ATG16L1 protein levels (Cadwell, Liu et al. 2008), while in murine macrophages ATG16L1 seems to regulate IL-1β secretion and inhibit intestinal inflammation (Saitoh, Fujita et al. 2008). Another study published in Nature by Murthy *et al*, showed that upon cellular stress and in the presence of the Ala281Thr variant, caspase 3 gets activated and ATG16L1 protein becomes more sensitive to cleavage, which may lead to impaired autophagy and subsequently to CD onset (Murthy, Li et al. 2014). Moreover, IRGM gene, which is also involved in autophagy (Singh, Davis et al. 2006), has been associated with CD risk (Wellcome Trust Case Control

Consortium 2007, Parkes, Barrett et al. 2007). Genetic variants within or upstream of the IRGM locus are linked to CD susceptibility (Parkes, Barrett et al. 2007, McCarroll, Huett et al. 2008, Rufini, Ciccacci et al. 2015), suggesting that autophagy pathways may play a key role in the development of Crohn's disease.

More recent genetic studies have identified TNFSF15 as another gene of the innate immune system to be associated with CD susceptibility (Jostins, Ripke et al. 2012, Franke, McGovern et al. 2010). TNFSF15 encodes for a protein named TL1A, which is a member of the TNF superfamily. TL1A is produced by innate immune cells such as monocytes, macrophages and DCs upon microbial stimulation and it binds to death domain receptor 3 (DR3) expressed on T cells, leading to T cell activation and secretion of proinflammatory cytokines (Migone, Zhang et al. 2002, Prehn, Thomas et al. 2007, Shih, Kwan et al. 2009). A SNP within the TNFSF15 gene has been strongly associated with CD susceptibility among Japanese, as well as East Asians and Europeans with IBD (Jostins, Ripke et al. 2012, Franke, McGovern et al. 2010, Yamazaki, McGovern et al. 2005, Kakuta, Kinouchi et al. 2006, Picornell, Mei et al. 2007, Thiebaut, Kotti et al. 2009, Tremelling, Berzuini et al. 2008, Yang, Lim et al. 2008). Interestingly, different haplotypes of the gene seem to be associated with either risk or protection depending on ethnicity (Picornell, Mei et al. 2007). In accordance with its genetic contributions, several pre-clinical studies have shown a role for TL1A in IBD pathogenesis (Barrett, Zhang et al. 2012, Shih, Barrett et al. 2011, Zheng, Zhang et al. 2013). Mice overexpressing TL1A develop spontaneous intestinal inflammation characterized by Th2 responses and high levels of IL-13 (Meylan, Song et al. 2011, Taraban, Slebioda et al. 2011), supporting the hypothesis of TLIA being an important regulator of mucosal immunity.

Components of the adaptive immune system have also been implicated with IBD susceptibility. Some of the strongest associations with increased CD risk found by GWAS are genetic variations within the gene coding for the IL-23R (Duerr, Taylor et al. 2006). IL-23R variants are also found in patients with UC, psoriasis and ankylosing spondylitis (Duerr, Taylor et al. 2006, Cargill, Schrodi et al. 2007, Wellcome Trust Case Control Consortium, Australo-Anglo-American Spondylitis Consortium (TASC) et al. 2007), supporting the notion of genetic overlap among these diseases. Most importantly, associations with CD susceptibility have been

reported for several genetic loci of the IL-23:IL-17A axis (Duerr, Taylor et al. 2006, Barrett, Hansoul et al. 2008), while protein levels of IL-23 and Th17-relevant cytokines are increased in the colonic mucosa of both CD and UC patients (Annunziato, Cosmi et al. 2007, Fujino, Andoh et al. 2003a, Saruta, Yu et al. 2007), highlighting the significance of this pathway in the pathogenesis of IBD. In particular, several genes of the IL-23 signalling pathway including JAK2, STAT3 and p40 have been associated with both forms of IBD (Barrett, Hansoul et al. 2008, Franke, Balschun et al. 2008b, Silverberg, Cho et al. 2009, Fernando, Stevens et al. 2008).

1.1.2 Environmental implications in IBD

Despite the accumulating evidence from GWAS strongly associating several genetic variants with IBD susceptibility, familial, and in particular twin studies, as well as epidemiological data clearly point out the need of additional factors for disease development. Such factors include diet, smoking, changes in the microbiome composition, certain types of surgery and geography. Overall, the relations between genetic predisposition and environmental influences remain unclear, highlighting the need of further studies to unravel the complicated and multifactor aetiology of IBD pathogenesis.

Dietary habits may affect IBD occurrence, although it is quite challenging to study those associations. Breast-feeding for longer than 6 months, high consumption of fibres and low sugar intake seems to reduce the possibility of IBD development (Hansen, Jess et al. 2011). In particular, fibre intake from fruits specifically appears to reduce CD risk to a greater extent compared to fibres form vegetables, and this association is stronger for small intestinal CD than colonic (Ananthakrishnan, Khalili et al. 2013, Amre, D'Souza et al. 2007). Daily consumption of vegetables and whole meal bread also reduce the odds for IBD occurrence (Jakobsen, Paerregaard et al. 2013). Omega 3 fatty acids that were thought to be beneficial for inflammatory disorders appear to have no effect on maintenance of remission in CD (Turner, Zlotkin et al. 2009).

Smoking is probably the best-studied environmental factor associated with IBD, and interestingly enough it seems to affect differently CD and UC risk. It increases the

risk of CD, whereas it protects against UC (Cosnes, Beaugerie et al. 2001, Lindberg, Tysk et al. 1988). Current smokers are more likely to develop CD than non-smokers (Mahid, Minor et al. 2006, Higuchi, Khalili et al. 2012), while CD patients that smoke are in greater risk of experience aggressive disease and need surgery (Cosnes, Carbonnel et al. 1996, Cosnes, Carbonnel et al. 1999, Cosnes 2004, Cosnes 2008). In contrast, ex-smokers have significantly increased risk of UC occurrence than non-smokers (Higuchi, Khalili et al. 2012). In people with already established UC, smoking is associated with milder disease, while smoking cessation is associated with flares (Beaugerie, Massot et al. 2001).

Emerging data point to vitamin D being associated with IBD occurrence. IBD incidence is suggested to be higher in northern latitudes and areas that are less exposed to UV light (Ananthakrishnan, Khalili et al. 2012). Moreover, both CD and UC patient's lower levels of vitamin D were associated with increased risk for surgery and IBD-related hospitalizations (Ananthakrishnan, Cagan et al. 2013).

Another environmental factor that appears to be associated with IBD risk include appendectomy, which is associated with risk for UC but not CD (Andersson, Olaison et al. 2001).

In an effort to understand the pathobiology of IBD, more than 60 animal modes of experimental inflammatory bowel disease have been described over the last decades. And in spite the fact that each and every one of them has its limitations (mostly in terms of resemblance to human disease), they helped us unravel several important aspects in the aetiology and pathophysiology of IBD including the involvement of the host's microbiome, its genetic background, as well as the role of the innate and adaptive immune system. In general, based on the induction of the disease these models can be divided into 5 different groups: (a) antigen-specific and bacterial models, (b) chemical/immunological and physical inducible models, (c) genetic models, (d) adoptive transfer models and (e) spontaneous models (Hoffmann, Pawlowski et al. 2002).

1.1.2.1 *Antigen-specific models*

This relatively small category includes among others the OVA TCR TG mouse model (Iqbal, Oliver et al. 2002), as well as *Helicobacter hepaticus* induced models

of experimental colitis. In the OVA TCR TG model, colitis is induced by transfer of either polarized Th1 or Th2 cells into *Rag2* KO mice colonized with OVA-expressing *E. coli* (Iqbal, Oliver et al. 2002). Although disease severity is similar between Th1 and Th2 transfers, histological features differ (Iqbal, Oliver et al. 2002). *Helicobacter hepaticus* induces intestinal inflammation in *Rag2* KO mice (von Freeden-Jeffry, Davidson et al. 1998). In this model, colitis is IL-7 dependent, as *Rag2xIl7* double KO mice do not develop disease (von Freeden-Jeffry, Davidson et al. 1998). *Helicobacter hepaticus* also induces colitis in mice deficient in the p50 chain of NF-κB and heterozygously deficient for the p65 chain (Erdman, Fox et al. 2001).

1.1.2.2 *Inducible models*

This group consists of animal models where the disease is induced chemically, immunologically or physically, including the first ever described animal model of experimental colitis, where intestinal inflammation in rabbits is caused by an immune complex (Kirsner 1961). Physically, intestinal inflammation can be induced via irradiation of MHC class II deficient mice (Marguerat, MacDonald et al. 1999). Amongst the chemically induced models, including oxazole colitis (Boirivant, Fuss et al. 1998) and the indomethacin model (Yamada, Deitch et al. 1993), the most commonly used are the dextran sodium sulphate (DSS) model (Cooper, Murthy et al. 1993) and the 2,4,6-trinitrobenzenesulfonic acid (TNBS) or dinitrobenzene sulfonic acid (DNBS) model (Morris, Beck et al. 1989).

Oral administration of 3-10% DSS via drinking water can cause colitis to several species such as mice, rats and hamsters that resembles some features of human UC (Okayasu, Hatakeyama et al. 1990, Kitajima, Takuma et al. 1999). Disease seems to be driven by direct epithelial damage leading to fibrosis, goblet cell hypoplasia and crypt loss, and is presented by weight loss, diarrhoea, rectal bleeding, shrinking of the colon and neutrophil infiltration (Okayasu, Hatakeyama et al. 1990, Kitajima, Takuma et al. 1999, Melgar, Karlsson et al. 2005). SCID and *Rag*$^{-/-}$ mice that lack B and T cells are also susceptible to DSS colitis, suggesting that adaptive immunity has no contribution at least to the acute phase of the disease (Dieleman, Ridwan et al. 1994, Krieglstein, Cerwinka et al. 2002). However, in chronic DSS colitis T cells that accumulate to the colon appear to be pathogenic (Dieleman, Palmen et al. 1998).

Whether or not microbiota play a role in DSS colitis is still unclear with several studies supporting either side. Although bacterial products and or probiotics can improve DSS colitis (Rachmilewitz, Katakura et al. 2004, Verdu, Bercik et al. 2000), germ-free mice seem to be equally or even more prone to disease (Bylund-Fellenius et al., Microbiol Ecol Health, 1994; Kitajima, Morimoto et al. 2001).

DNBS/TNBS colitis is induced by intrarectal administration of DNBS/TNBS dissolved in ethanol (EtOH) that results in the generation of Ag-specific adoptive immune responses (Elson, Beagley et al. 1996). However, innate immune cells are also involved as DNBS/TNBS inflammation also occurs in mice lacking lymphocytes (Fiorucci, Mencarelli et al. 2002). DNBS/TNBS inflammation seems microbiota and strain dependent with distinct cytokine profiles (Th1, Th2, Th17) being described in different genetic backgrounds (Elson, Beagley et al. 1996, Neurath, Fuss et al. 1995a, Dohi, Fujihashi et al. 1999). DNBS/TNBS induced inflammation shares many similarities with CD pathology, including the persistent fibrosis observed in the chronic version of this model (Kiesler, Fuss et al. 2015, Fichtner-Feigl, Fuss et al. 2007).

1.1.2.3 *Genetic models*

This is the biggest and most growing category of IBD animal models including both transgenic and knock-out (KO) animal models. In general, genetic models can be further divided into 5 groups based on the pathway that is altered: (a) models with IL-2/IL-2R deficiencies, (b) models with IL-10 signalling deficiencies, (c) TCR or MHC class II deficient models, (d) TGFβ deficient models and (e) models with alterations in signal transduction molecules (A20, Gai2, STAT-3, STAT-4) (Hoffmann, Pawlowski et al. 2002).

One of the best-characterized and commonly used genetic models is the IL-10 deficient mouse. In this model, disease is very much dependent on T cells (Davidson, Leach et al. 1996). IFNγ signalling is also important as administration of neutralizing antibodies against IFNγ in young IL-10 deficient mice abrogates intestinal inflammation (Berg, Davidson et al. 1996). The same results are observed with administration of recombinant IL-10 (Rennick, Fort et al. 1997). In adult IL-10 deficient mice, colitis onset can be prevented by anti-IL-12 Ab administration (Davidson, Hudak et al. 1998). Intestinal microbiota also mediate inflammation in

IL-10 deficient mice (Rennick, Fort et al. 1997). Germ-free IL-10 deficient mice do not develop colitis unless introduced to specific bacterial flora (Sellon, Tonkonogy et al. 1998). *Helicobacter hepaticus* (HT) seems able to induce colitis in IL-10 deficient mice (Kullberg, Ward et al. 1998), although other studies support that it has no effect in disease induction (Dieleman, Arends et al. 2000). With regards to human disease, IL-10 deficient mice develop colon cancer similarly to CD and UC patients (Sturlan, Oberhuber et al. 2001), as well as osteoporosis like CD patients do (Sylvester, F et al. 2002).

1.1.2.4 *Adoptive transfer models*

This group comprises all IBD animal models where disease is induced by adoptive transfer of naïve T cells into immunodeficient recipients (*Rag1* KO, *Rag2* KO, SCID mice). Adoptively transferred $CD4^+CD45RB^{high}$ T cells into SCID mice lead to colitis (Powrie, Leach et al. 1993, Morrissey, Charrier et al. 1993), as do $CD4^+CD62L^+$ T cells (Mudter, Wirtz et al. 2002, Neurath, Weigmann et al. 2002). In general, all T helper (Th) cells can cause colitis if adoptively transferred to immunocompromised recipients (Claesson, Bregenholt et al. 1999). Inflammation seems to be driven by Th1 responses as *in vivo* blocking of IFNγ, TNFα and IL-12 but not IL-4 prevents disease development (Brimnes, Reimann et al. 2001, Powrie, Leach et al. 1994, Leach, Bean et al. 1996, Mackay, Browning et al. 1998), while dendritic cells (DCs) are also important mediators (Leithauser, Trobonjaca et al. 2001, Malmstrom, Shipton et al. 2001). Interestingly, in this model(s) disease development can be prevented if $CD4^+CD45RB^{low}$ T cells are co-transferred with $CD4^+CD45RB^{high}$ T cells (Powrie, Leach et al. 1993, Morrissey, Charrier et al. 1993, Groux, O'Garra et al. 1997), an effect that is mediated by IL-10 production (Asseman, Mauze et al. 1999a). Similarly, colitis induction is abrogated by *in vivo* administration of anti-TGFβ (Powrie, Carlino et al. 1996) or anti-IL-10R1 Abs (Asseman, Mauze et al. 1999b).

As observed in other IBD animal models, disease induction here is also driven by the intestinal microbiota. If $CD4^+CD45RB^{high}$ T cells are transferred to SCID recipients with impaired flora or to mice treated with antibiotics, disease is ameliorated (Aranda, Sydora et al. 1997). Moreover, polyclonal $CD4^+CD45RB^{high}$ T cells become oligoclonal and Ag-reactive once transferred (Matsuda, Gapin et al. 2000).

Finally, pathology in these models share many similarities with that observed in UC patients (Leach, Bean et al. 1996).

1.1.2.5 *Spontaneous models*

Animals rarely develop spontaneous colitis. Murine models in this category include the SAMP1/Yit mice that under SPF conditions spontaneously develop intestinal inflammation with clinical features similar to CD (Matsumoto, Okabe et al. 1998), and the C3H/HeJ mice that can also develop colitis under certain housing conditions (Sundberg, Elson et al. 1994).

Another model of spontaneous IBD that resembles human UC was firstly described by Garrett *et al,* in an article published in Cell, back in 2007. Tbet deficiency in the innate immune compartment leads to spontaneous colitis in the absence of adaptive immunity (Garrett, Lord et al. 2007). *Tbx21*$^{-/-}$ *x Rag2*$^{-/-}$ mice spontaneously develop severe colitis with features similar to those observed in UC patients, hence referred to as *Tbx21*$^{-/-}$ *x Rag2*$^{-/-}$ Ulcerative colitis (TRUC) mice (Garrett, Lord et al. 2007). Disease is presented as early as 4 weeks with continuous inflammation starting from the rectum and followed by the left colon, intestinal wall thickening and anal prolapse, while the remaining gastrointestinal tract appears normal (Garrett, Lord et al. 2007). Histologically, is characterized by mononuclear, polymorphonuclear cell infiltration, goblet cell depletion, epithelial injury, and crypt loss (Garrett, Lord et al. 2007). TRUC disease is driven by TNFα overproducing colonic DCs (Garrett, Lord et al. 2007), and at the early stages it can be ameliorated by anti-TNFα neutralizing Abs; anti-TNFα treatment is ineffective on 12-week old or older mice (Garrett, Punit et al. 2009). Similarly to other IBD animal models, the intestinal microflora has a key role in mediating TRUC disease (Garrett, Lord et al. 2007), as germ-free TRUC mice do not develop colitis (Garrett, Gallini et al. 2010) and inflammation is resolved following treatment with antibiotics (Garrett, Lord et al. 2007, Garrett, Gallini et al. 2010) or probiotics (Veiga, Gallini et al. 2010). Moreover, TRUC disease is communicable to Tbet sufficient mice (Garrett, Lord et al. 2007).

Recent work in our lab by Powell *et al* shed more light into the immunopathology of TRUC disease. In particular, IL-17A producing IL-7R^{+} innate lymphoid cells (ILCs) were proven to be key mediators of chronic colitis in the TRUC model of IBD (Powell, Walker et al. 2012). *In vivo* administration of anti-CD90 neutralizing Abs

significantly reduced intestinal inflammation, and the same effects were observed with IL-17A blockade (Powell, Walker et al. 2012). IL-17A production by ILCs is induced by IL-23 and TNFα, which is excessively produced by $CD103^{-}CD11b^{+}$ colonic DCs (Powell, Walker et al. 2012). Moreover, IL-7R signalling is also crucial for TRUC pathology as *in vivo* inhibition of IL-7R signalling seems to ameliorate disease (Powell, Walker et al. 2012). Most importantly, Powell *et al* identified *Helicobacter typhlonius* as the main microbiota component that triggers TRUC disease (Powell, Walker et al. 2012).

More recently, an independent study by Ermann et al corroborated those findings, while identifying NOD2 as an important upstream regulator of IL-23/IL-1 induced IL-17A production by ILCs, and TNFα as a key inducer of neutrophil chemo-attractants (Ermann, Staton et al. 2014).

Table 1: Available animal models to study IBD. (Modified from Hoffmann JC *et al*., 2002). All models highlighted in maroon were used for the work described in this thesis.

Antigen-specific	Inducible	Genetic	Adoptive transfer	Spontaneous
	Chemical	***Transgenic***		
Peptidoglycan-polysaccharide[r]	Acetic acid[r,d]	Cytochrome c TCR tg[m]	CD4+CD45RB^{high}/SCID[m]	C3H/HeJBir[m]
Carageenan[m, gp, rb]	**DNBS/ethanol[m,r]**	HGF tg[m]	CD4+CD45RB^{high}/RAG-2 ko[m]	Cotton-top tamarin[p]
Complete Freund's adjuvant[r]	**DSS[h,m,r]**	HLA B27 x β_2-MG tg[r]	CD4+CD62L^{high}/SCID[m]	Grower/finisher[pgs]
Helicobacter hepaticus (rag-2 ko)[m]	Indomethacin[r]	HSV tyrosinekinase tg/gancyclovir[m]	**CD4+CD62L^{high}/RAG-1 ko[m]**	HistiocyticUC[d]
Helicobacter hepaticus (p50$^{-/-}$ x p65$^{+/-}$)[m]	Lactulose[m]	HTF tg[m]	CD4+CD45RB^{high}Yit/SCID[m]	Samp1/Yit[m]
Hsp60[m]	Oxazolone[m]	IL-7 tg[m]	CD4+/SCID[m]	
Lymphogranuloma venereum proctitis[m]	Sulfhydryl blockers[r]	N-Cadherin dominant neg. tg[m]	CD4+ con A blasts/SCID[m]	
Ovalbumin in OVA TCR tg mice[m]	TNBS/ethanol[r,m,rb]	STAT4 tg/TNP-KLH[m]	CD4+ con A blasts/RAG-1 ko[m]	
Ovalbumin/transfercolitis/OVA tg mice[m]		TGFβ receptor-II dominant neg. tg[m]	Bone marrow/CD3$_\varepsilon$Tg26[m]	
TNBS/transfer/TCR ko[m]	***Immunological***	CD40L tg[m]	HSP60-CD8 clone/TCRβ ko[m]	
	Cyclosporin A[m]		MuLV splenocytes/nude[m]	
	Immune complex[rb,r]	***Knock-out***		
		A20 ko[m]		
	Physical	Iκβ$_\alpha$ ko[m]		
	Radiation (MHC II ko)[m]	Gi-$_{2\alpha}$ ko[m]		
		Gpx 1 ko x Gpx 2 ko[m]		
		IL-2 receptor-β ko[m]		
		IL-2 receptor-α ko[m]		
		IL-2 ko x β_2-MG ko[m]		
		IL-2 ko[m]		
		IL-10 ko[m]		
		CRF2-4 ko[m]		
		Mdr1a ko[m]		
		MHC class-II ko[m]		
		Myeloid STAT 3 ko[m]		
		Smad3 ko[m]		
		TCR-α ko[m]		
		TCR-β ko[m]		
		TGF-β ko[m]		
		TGF-βRII ko[m]		
		TNF$^{\Delta ARE\ m}$		
		WASP ko[m]		
		TRUC[m]		

Indicated are the category and the species (d=dog; gp+guinea pig; m=mouse; p=primate; pgs=piglets; r=rat; rb=rabbit). DSS=Dexxtran sulfonic acid; HGF= hepatocyte growth factor; HTF= human fucosyl transferase; mdr=multiple drug resistance gene; tg=transgenic; TGF=transforming growth factor

1.2 Immunopathology of IBD

Although the exact cause of IBD remains unclear, accumulating evidence suggest that it results from a dysregulated immune response against the normal intestinal microbiota in a genetically predisposed host. Recent genetic studies associating SNPs at genetic loci involved in host detection, processing and presentation of luminal bacteria with altered IBD risk highlight the importance of host-microbe interactions in IBD pathogenesis. Similarly, studies showing how changes in microbiota composition, due to environmental factors such as diet and treatment with antibiotics, affect IBD occurrence also support this hypothesis. Extensive studies focusing on microbe-host interactions and the multiple functions of the mucosal immune system in the gut have provided new insights into both etiology and pathobiology of IBD.

1.2.1 The microbiome

The human gastrointestinal tract with a luminal surface of around $300m^2$ provides home to more than 10^{13} microorganisms (Gill, Pop et al. 2006). This enormous microbial population that can be quite diverse even among healthy individuals (Human Microbiome Project Consortium 2012), helps us get the most of our dietary intake by complementing our metabolic capacity (Backhed, Ley et al. 2005), and has a great impact on shaping our intestinal immune responses. A person's intestinal microbiota is acquired at birth but changes during the first year of life, while it remains quite stable throughout adulthood, although its composition may alter slightly in response to environmental factors or during disease (Eckburg, Relman 2007, Turnbaugh, Hamady et al. 2009, Wu, Estrada et al. 2005). Several studies have now mapped the "normal" human gut microbiome, which is shared among most healthy individuals. The two dominant phyla are *Bacteroidetes* and *Firmicutes*, whereas *Actinobacteria*, *Proteobacteria, Fusobacteria* and *Verrucomicrobia* are also found in some people (Eckburg, Bik et al. 2005), as well as Methanogenic archaea, eukaryotes and viruses (Reyes, Haynes et al. 2010). However, the specific species of those phyla and their relative proportions differ notably among individuals.

Patients with IBD have altered microbiota composition (Frank, St Amand et al. 2007, Ott, Musfeldt et al. 2004). Twins with UC were found to have less *Bacteroides* and more *Actinobacteria* and *Proteobacteria* compared to their healthy twin siblings (Lepage, Hasler et al. 2011). *E. coli* has also been found to be increased in people

with UC, while is also found in patients with CD (Chassaing, Darfeuille-Michaud 2011). On the other hand, patients with CD appear to have less bacterial numbers of the phylum *Firmicutes* than healthy individuals (Kang, Denman et al. 2010, Manichanh, Rigottier-Gois et al. 2006). Moreover, altered bacterial composition has also been observed between CD patients, whose disease affects the ileum and colon (Willing, Dicksved et al. 2010). Taken together these data support the concept of microbiota having an important role in IBD pathobiology by providing the initial stimulus for an inflammatory immune response.

1.2.2 The intestinal epithelium

The epithelial cells of the gastrointestinal tract provide a physical barrier that separates the microbiota normally residing in the lumen, from the immune cells of the lamina propria, ensuring homeostasis. Only a small portion of bacteria is able to translocate to allow "normal" bacterial sampling by cells of the innate immune system (Slack, Hapfelmeier et al. 2009). Defects in the intestinal barrier leading to increased permeability and subsequently to persistent immune activation may lead to IBD (Sartor 2006). The intestinal epithelium consists of five functionally distinct cell types that derive from a common stem cell: 1) Absorptive enterocytes, 2) Mucus producing goblet cells, 3) hormone producing enderoendocrine cells, 4) M cells and 5) antimicrobial and growth factor producing Paneth cells (van der Flier, Clevers 2009).

Goblet cells produce and secrete glycosylated mucins forming the mucosa (just above the epithelium), which comprises the first line of defence against the microbes in the lumen (Johansson, Ambort et al. 2011). The colon has a dual mucus layer restricting bacterial adhesion to the epithelium (Johansson, Larsson et al. 2011); whereas the small intestinal has a looser single layer of mucus allowing penetration of food substances (Sonnenburg, Xu et al. 2005). Paneth cells are located at the bottom of the crypts and are known for producing antimicrobial peptides (AMPs) and other inflammatory mediators (Bevins, Salzman 2011). Several genetic variants that are associated with IBD risk including those within the NOD2 locus affect Paneth cell function (Lala, Ogura et al. 2003), while goblet cell and mucus depletion are characteristics of UC (Jass, Walsh 2001, Danese, Fiocchi 2011), suggesting a role of the epithelium in IBD pathogenesis.

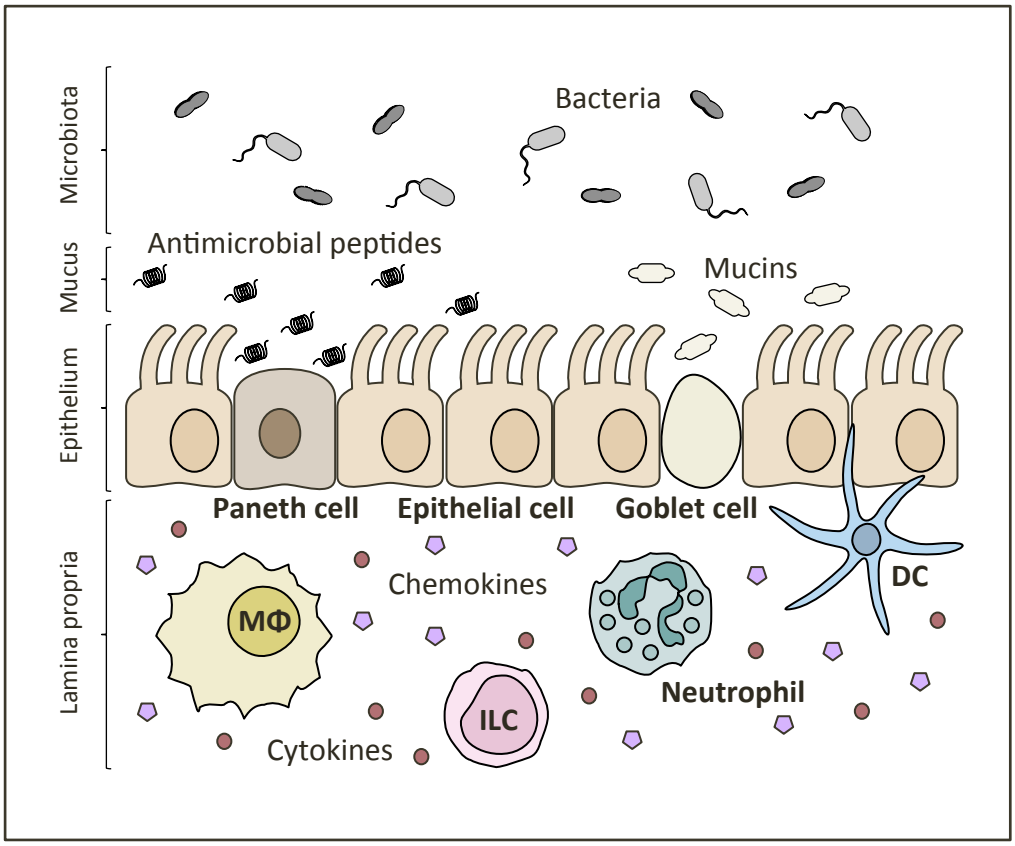

Figure 1: Innate immune responses in the gut. The intestinal epithelium forms a physical anatomical barrier that separates the lumenal contents from the rest of our body. It consists of several types of epithelial cells including antimicrobial peptide-producing Paneth cells, and mucin-secreting Goblet cells that form a mucus layer protecting the epithelium from direct contact with bacterial antigens. Right beneath the epithelium lays a thin layer of connective tissue known as *lamina propria*, enriched with innate immune cells such as macrophages, DCs, neutrophils and ILCs. In steady state, a limited translocation of commensal bacteria from the lumen to the LP is allowed, where mononuclear phagocytes sample bacterial antigens through TLRs and NLRs without initiating an inflammatory response, therefore securing intestinal homeostasis.

1.2.3 The intestinal immune system

A complex network of interactions exists among the microbiome, the epithelium and the immune cells that reside along the walls of the gastrointestinal tract. The intestinal immune system has been assigned with the difficult task of discriminating between commensal, harmless bacteria and invading pathogens that translocate across the epithelial monolayer, while is trained to maintain tolerance against the first and initiate protective immune responses against the later securing intestinal homeostasis. Breakdown of this fine balance between the host and its intestinal microbiota can lead to intestinal inflammation and subsequently to development of IBD.

1.2.3.1 *Mononuclear phagocytes (MPs)*

The intestinal mononuclear phagocyte (MP) system consists of two functionally distinct cell types: Mφs (the main phagocytes) and DCs (the professional antigen-presenting cells), both of which play a central role in antigen sampling and T cell priming. Despite their apparent functional differences, these cells have a similar phenotype, which makes it difficult to differentiate them based on their surface markers, leaving their subtype identification somewhat controversial.

- Intestinal Mϕs

In contrast to other tissues, intestinal Mφs are constantly replenished by blood circulating monocytes (Tamoutounour, Henri et al. 2012, Bain, Scott et al. 2013, Rivollier, He et al. 2012, Bain, Bravo-Blas et al. 2014). In particular, $Ly6C^{hi}$ monocytes continuously arrive to the intestines in a CCR2-dependent manner (Bain, Bravo-Blas et al. 2014), where they start to down- regulate Ly6C while they start to up-regulate MHC II, CD64 and F4/80 through a process known as the monocyte waterfall (Tamoutounour, Henri et al. 2012). Ultimately, they give rise to resident Mφs that also express high levels of the chemokine receptor CX3CR1 (Tamoutounour, Henri et al. 2012, Bain, Scott et al. 2013) and can be identified as $Ly6C^{-}CD11c^{-}MHCII^{+}CD11b^{+}CD64^{+}$ cells. They can be found throughout the gastrointestinal tract, mostly close to the epithelium (Hume, Perry et al. 1984), however their numbers tend to increase in the colon (in comparison with the small intestine) reaching their zenith towards the distal end (Nagashima, Maeda et al. 1996,

Denning, Norris et al. 2011). A subpopulation of CX3CR1 expressing Mφs is located close to the epithelium that reaches the intestinal lumen to sample antigens (Niess, Adler 2010). Mice lacking CX3CR1 have less Mφs in the lamina propria, high translocation of commensal bacteria to the MLN, and develop severe colitis, which can be ameliorated by IL-17A blockade or adoptive transfer of CX3CR1 sufficient Mφs (Medina-Contreras, Geem et al. 2011).

In steady state mucosa, macrophages exhibit an anti-inflammatory phenotype by producing IL-10 and low levels of TNFα (Bain, Scott et al. 2013), showing poor response to TLR ligands (Tamoutounour, Henri et al. 2012, Bain, Scott et al. 2013) and lacking respiratory burst activity (Rugtveit, Haraldsen et al. 1995) or nitric oxide production (Roberts, Riley et al. 2001). However, intestinal Mφs are highly phagocytic (Smith, Smythies et al. 2011, Smythies, Sellers et al. 2005, N'Diaye, Branda et al. 2009). Their postulated roles include maintenance of epithelial turnover/integrity and tissue remodeling (Muller, Kaiser et al. 2012, Rani, Smulian et al. 2011, Pull, Doherty et al. 2005). They have also been implicated in the differentiation and maintenance of the $FoxP3^+$ Treg pool (Hadis, Wahl et al. 2011, Murai, Turovskaya et al. 2009) and T effector cell lineages (Shaw, Kamada et al. 2012). Similarly, in humans, intestinal Mφs lack CD14 expression and under normal conditions exhibit anti-inflammatory properties, whilst remaining highly phagocytic (Smythies, Sellers et al. 2005, Smith, Smythies et al. 2001). However, in CD patients, an additional Mφ population is present that expresses CD14 as well as other Mφ and DC markers such as CD33, CD68, CD205 and CD209 and produces high levels of pro-inflammatory cytokines including TNFα, IL-6 and IL-23 (Kamada, Hisamatsu et al. 2008). These Mφs seem to regulate IFNγ, but not IL-17A production by LPMCs (Kamada, Hisamatsu et al. 2008).

During intestinal inflammation, instead of giving rise to anti-inflammatory Mφs, newly recruited $Ly6C^{hi}$ monocytes give rise to $CX3CR1^{int}$ expressing pro-inflammatory Mφs (Bain, Scott et al. 2013, Zigmond, Varol et al. 2012, Weber, Saurer et al. 2011). Many independent studies using experimental models of colitis based on DSS or naïve T cell transfer, suggest that in this context, $Ly6C^{hi}$ monocytes and their derivatives are highly pathogenic (Zigmond, Varol et al. 2012, Platt, Bain et al. 2010, Varol, Vallon-Eberhard et al. 2009).

- Intestinal DCs

Intestinal DCs are generally identified as MHCII^{hi}CD11c^{hi}CD64^{-}F4/80^{-} cells that can subsequently be separated into four functionally distinct subsets based on their expression of CD103 and CD11b. CD103^{+}CD11b^{+} DCs comprise the majority of CD103^{+} DCs in the small intestine LP, but are rare in the colonic LP and their development depends on Flt3 (as of most classical DCs) (Bogunovic, Ginhoux et al. 2009), GM-CSF (Greter, Helft et al. 2012), Notch-2 (Lewis, Caton et al. 2011) and IRF4 (Schlitzer, McGovern et al. 2013, Persson, Uronen-Hansson et al. 2013). In steady state, these cells represent the main compartment of CD103^{+} DCs that induces tolerance, and although their absence doesn't affect FoxP3^{+} generation it decreases the number of Th17 cells (Lewis, Caton et al. 2011, Persson, Uronen-Hansson et al. 2013, Welty, Staley et al. 2013). However, upon TLR5 activation CD103^{+}CD11b^{+} DCs can prime T cells suggesting that these cells can switch from an anti-inflammatory (tolerogenic) phenotype to a pro-inflammatory (immunogenic) one (Kinnebrew, Buffie et al. 2012, Uematsu, Fujimoto et al. 2008).

CD103^{+}CD11b^{-} DCs, whose development depends on Flt3, Batf3 and IRF8, share ontogeny as well as function with splenic CD8α^{+} DCs (Edelson, KC et al. 2010). They are able to cross- present antigen priming CD8^{+} T cells (Cerovic, Houston et al. 2013, Fujimoto, Karuppuchamy et al. 2011), while they produce ALDH in MLN suggesting that they may play a role in FoxP3^{+} Treg induction (Cerovic, Jenkins et al. 2009). During inflammatory conditions, CD103^{+} DCs in MLN respond to TLR signaling and induce IFNγ production by CD4^{+} and CD8^{+} T cells (Cerovic, Houston et al. 2013, Cerovic, Jenkins et al. 2009).

CD103^{-}CD11b^{+} DCs are difficult to distinguish from intestinal Mφs as they share phenotypic characteristics including intermediate expression levels of CX3CR1. Their progenitors and functions are poorly understood, although they are not Ly6C^{hi} monocyte derived and expand upon Flt3 treatment. Like other DC subsets, CD103^{-} CD11b^{+} DCs are found in small intestine and colonic LP as well as in MLN (Bain, Scott et al. 2013, Cerovic, Houston et al. 2013) and are capable of priming T cells (Cerovic, Houston et al. 2013). Lastly, CD103^{-}CD11b^{-} DCs are the minority of intestinal DCs and rare in the colonic LP. Their development may depend on Flt3 as

they proliferate after Flt3 administration, whereas they can induce IL-17 production by $CD4^+$ T cells *in vitro* (Cerovic, Houston et al. 2013).

Under inflammatory conditions (e.g. in experimental models of colitis) DCs accumulate in the gut (Uhlig, McKenzie et al. 2006), as well as in the mucosa of patients with IBD (Hart, Al-Hassi et al. 2005). In TRUC mice that develop spontaneous colitis TNFα producing $CD103^-CD11b^+$ DCs promote intestinal inflammation (Powell, Walker et al. 2012). A DC subset that expresses E-cadherin also promotes intestinal inflammation in experimental models of colitis via IL-6 and IL-23 production (Siddiqui, Laffont et al. 2010).

1.2.3.2 *Lymphocytes*

Adaptive immune cells, and in particular exaggerated T cell responses have also been associated with IBD pathogenesis. Initially, it was thought that CD is a Th1 mediated disease as T cells isolated from the mucosa of CD patients produce high amounts of IFNγ and IL-2 (Breese, Braegger et al. 1993, Noguchi, Hiwatashi et al. 1995), while UC is characterized by a Th2 immune response with UC patients producing more IL-5 and IL-13 than individuals with CD (Fuss, Neurath et al. 1996, Fuss, Heller et al. 2004, Heller, Florian et al. 2005). However, the fact that higher levels of IFNγ and IL-13 are found in the supernatants of cultures of biopsies obtained from UC patients when compared to those isolated from patients with CD (Rovedatti, Kudo et al. 2009, Vainer, Nielsen et al. 2000), challenge this paradigm. In addition, more recent studies implicate Th17 in IBD pathology, and in particular with CD, which is also consistent with all the GWAS that associate SNPs in the IL-23:IL-17A axis with increased IBD risk.

Neutralizing antibodies against IL-12, a cytokine known for inducing Th1 differentiation, have been shown to ameliorate disease in models of experimental colitis (Neurath, Fuss et al. 1995b). Accordingly in humans, anti-IL-12p40 antibodies (known as ustekinumab and briakinumab) seem to be effective in CD patients that do not respond to anti-TNF treatment (Sandborn, Gasink et al. 2012, Mannon, Fuss et al. 2004), suggesting a role for IL-12 in IBD pathology. However, IL-23, which shares the p40 chain with IL-12, is also blocked by the above drugs complicating the exact mechanisms of their action.

Th17 cells are known to be induced by IL-6 and TGFβ, expand by IL-23, and they produce high amounts of IL-17A, IL-17F, IL-21 and IL-22 (Dong, Nurieva 2003, Dong 2008, Zhou, Ivanov et al. 2007). Their differentiation depends on the transcription factor RORγt (Ivanov, McKenzie et al. 2006, Yang, Pappu et al. 2008), and they can be divided into two subsets based on their function with the IL-17A/IFNγ coproducing generally considered as the "pathogenic" Th17 subpopulation. IBD patients have increased IL-17A mRNA levels, as well as increased numbers of Th17 and Th1/Th17 cells in the lamina propria (Rovedatti, Kudo et al. 2009, Fujino, Andoh et al. 2003b, Sugihara, Kobori et al. 2010). Moreover, IL-17RA KO mice were protected from disease in a TNBS model of experimental colitis (Zhang, Zheng et al. 2006), also supporting a pathogenic role for IL-17A in IBD. In contrast, several studies have shown data suggesting a protective role for IL-17 in IBD pathogenesis. In a DSS model of colitis, IL-17A was found to be protective against mucosal inflammation, while IL-17F was shown to exacerbate disease (Moseley, Haudenschild et al. 2003, O'Connor, Kamanaka et al. 2009, Sarra, Pallone et al. 2010). Furthermore, blockade of IL-17A exacerbated disease in a DSS model of colitis (Ogawa, Andoh et al. 2004). Taken together these data leave unclear the implications of IL-17 in the pathobiology of IBD, while leaving open the possibility that genetic and/or other environmental factors affect the action of this particular cytokine in the context of intestinal inflammation.

1.3 Innate lymphoid cells

Beside the well-known lymphocytes and the classic innate immune cells such as macrophages and DCs, a new family of innate immune cells is emerging as key players in IBD. Recently discovered innate lymphoid cells, also known as ILCs, comprise phenotypically and functionally distinct, non-cytotoxic cells of the innate immune system (Spits, Di Santo 2011, Spits, Artis et al. 2013, Eberl 2012). The ILC superfamily also includes the well-described natural killer (NK) cells, as well as the lymphoid tissue inducer (LTi) cells (Spits, Artis et al. 2013). All ILCs are characterized by a lymphoid-like morphology as given away by their name, and the lack of antigen specific receptors, therefore exhibiting no antigen specificity (Spits, Di Santo 2011, Spits, Artis et al. 2013, Eberl 2012). In addition, although ILCs express IL-2Rα (CD25) and IL-7R (CD127), they don't express any surface markers that identify other immune subsets, instead they are defined by the lack thereof as Lineage negative (Lin^-) cells (Spits, Di Santo 2011, Spits, Artis et al. 2013, Eberl, Marmon et al. 2004, Mebius, Rennert et al. 1997, Moro, Yamada et al. 2010a, Neill, Wong et al. 2010, Price, Liang et al. 2010). ILCs are distributed throughout the body, in lymphoid as well as non-lymphoid tissues, and they can directly interact with numerous different cell types haematopoietic and non, being involved in several biological processes including the regulation of intestinal homeostasis (Spits, Di Santo 2011, Spits, Artis et al. 2013, Eberl 2012).

1.3.1 ILC development & heterogeneity

In contrast to T and B cells, the development of ILCs is yet to be fully characterized. Recent studies showed that ILC precursors (often referred to as α-lymphoid precursors) also derive from the common lymphoid progenitor (CLP), express the integrin α4β7 and the chemokine receptor CXCR6, and may give rise to conventional NK cells, as well to all ILC subsets (Klose, Flach et al. 2014, Cherrier, Sawa et al. 2012, Possot, Schmutz et al. 2011). Downstream of αLPs are two known precursors both expressing the transcriptional repressor Id2, which give rise to conventional NK cells and ILCs (Klose, Flach et al. 2014, Cherrier, Sawa et al. 2012, Constantinides, McDonald et al. 2014). Mice deficient in Id2 have neither NK cells nor ILCs (Moro, Yamada et al. 2010a, Cherrier, Sawa et al. 2012, Monticelli, Sonnenberg et al. 2011a, Hoyler, Klose et al. 2012a, Yokota, Mansouri et al. 1999). The $Id2^+$ ILC precursor

gives rise to all ILCs and LTi cells, but not to conventional NK cells (Klose, Flach et al. 2014). Next in line is the $Id2^+$ ILC precursor that expresses both Id2 and the transcriptional factor promyeloid leukaemia zinc finger (PLZF), and can only give rise to all ILC subsets but not to LTi cells (Constantinides, McDonald et al. 2014). Additional transcriptional factors that seem to affect early differentiation of NK cells and ILCs are TOX and NFIL3 (Constantinides, McDonald et al. 2014, Fuchs, Vermi et al. 2013, Aliahmad, de la Torre et al. 2010, Geiger, Abt et al. 2014, Seillet, Rankin et al. 2014, Yu, Wang et al. 2014), and the basic-helix-loop-helix transcriptional factor E47, which is reported to block the development of NK and LTi cells (Boos, Yokota et al. 2007), hence favouring ILC development.

Similarly to all T cell populations, ILC subsets are defined based on their need for transcriptional factors during development, the distinct combinations of cytokines they produce as well as their effector functions. Two important transcriptional factors that seem to drive the development of all ILCs that express the IL-7R (also known as CD127) are CTF-1 and GATA-3.

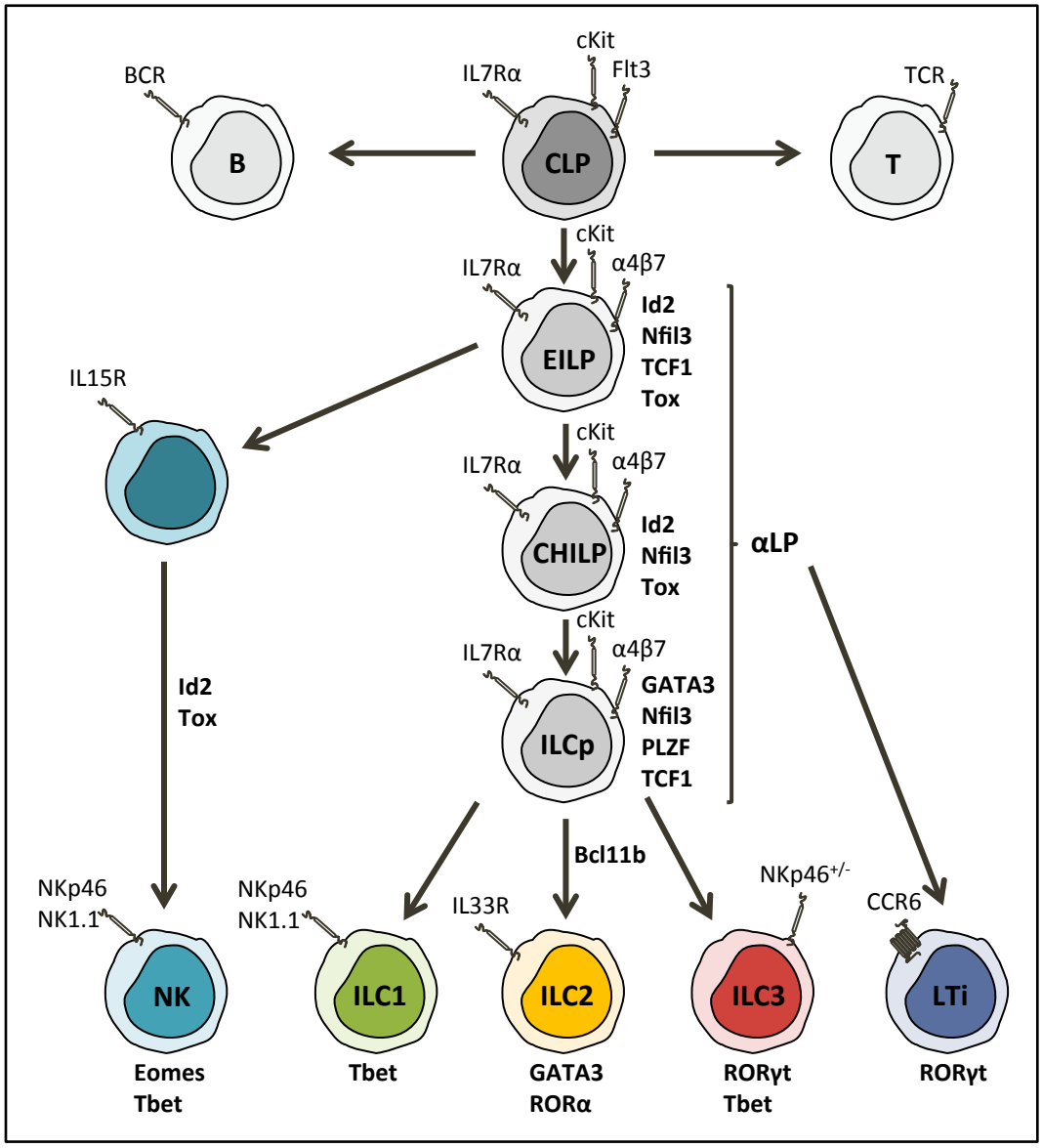

Figure 2: ILC development. A common lymphoid progenitor gives rise to an early ILC progenitor (EILP) in bone marrow and fetal liver. EILP can give rise to all ILC lineages, LTi cells and NK cells, but not to T or B cells. Downstream of EILP is the IL-15R-expressing NK precursor that gives rise to mature NK cells, as well as the common-helper like ILC precursor (CHILP) that is characterized by the expression of c-Kit, IL-7Rα, α4β7 and the transcription factors Id2, Tox and Nfil3. CHILP can further develop to LTi cells, or to PLZF-expressing precursor that gives rise to ILC1s, ILC2s and ILC3s, but not to LTi cells.

1.3.1.1 *Group 1 ILCs (ILC1s and NK cells)*

The group 1 ILCs comprises the well-known NK cells and the non-cytotoxic Lin^- ILC1s (Spits, Artis et al. 2013). NK cell development depends on the transcriptional factors T-bet and Eomes (Gordon, Chaix et al. 2012); whereas the development of ILC1s appears to be associated with a down-regulation of the transcriptional factor RORγt and an up-regulation of T-bet (Vonarbourg, Mortha et al. 2010a, Bernink, Peters et al. 2013b). Both NK cells and ILC1s are activated by the cytokines IL-12, IL-15 and IL-18 (Klose, Flach et al. 2014, Fuchs, Vermi et al. 2013, Bernink, Peters et al. 2013b) and produce high levels of IFNγ (Klose, Flach et al. 2014, Fuchs, Vermi et al. 2013, Vonarbourg, Mortha et al. 2010a, Bernink, Peters et al. 2013b). Alongside IFNγ, ILC1s are also known to produce TNFα (Klose, Flach et al. 2014). Several independent studies focused on ILC1s, have highlighted the importance of these cells in immunity against intracellular bacteria and parasites (Klose, Flach et al. 2014, Fuchs, Vermi et al. 2013, Vonarbourg, Mortha et al. 2010a, Bernink, Peters et al. 2013b).

1.3.1.2 *ILC2s*

In contrast to group 1 ILCs, ILC2s depend on the transcriptional factor GATA-3 for their development and maintenance (Hoyler, Klose et al. 2012b, Mjosberg, Bernink et al. 2012), while they also require IL-7 (Moro, Yamada et al. 2010b). Like Th2 cells, ILC2s respond to type 2 cytokines such as IL-2 (Mirchandani, Besnard et al. 2014, Oliphant, Hwang et al. 2014), IL-4 (Kim, Wang et al. 2014, Motomura, Morita et al. 2014), IL-25 and IL-33 (Neill, Wong et al. 2010, Price, Liang et al. 2010, Monticelli, Sonnenberg et al. 2011a, Moro, Yamada et al. 2010b, McHedlidze, Waldner et al. 2013, Imai, Yasuda et al. 2013, Saenz, Siracusa et al. 2013, Salimi, Barlow et al. 2013). Several studies have also shown that ILC2s get activated by IL-9 (Wilhelm, Hirota et al. 2011, Turner, Morrison et al. 2013), thymic stromal lymphopoietin (TSLP) (Mjosberg, Bernink et al. 2012, Kim, Siracusa et al. 2013) and TL1A (Meylan, Hawley et al. 2014, Yu, Pappu et al. 2014). Upon activation, ILC2s are shown to produce type 2 cytokines to mediate their functions like IL-4, IL-5, IL-9 and IL-13 (Neill, Wong et al. 2010, Price, Liang et al. 2010, Monticelli, Sonnenberg et al. 2011a, Moro, Yamada et al. 2010b, Wilhelm, Hirota et al. 2011, Mjosberg, Trifari et al. 2011). ILC2s have been shown to have an important role in

several physiological and pathological processes including tissue repair, helminth immunity allergies and asthma (Neill, Wong et al. 2010, Price, Liang et al. 2010, Monticelli, Sonnenberg et al. 2011a, Moro, Yamada et al. 2010b, Wilhelm, Hirota et al. 2011, Mjosberg, Trifari et al. 2011, Bartemes, Kephart et al. 2014, Moffatt, Gut et al. 2010). In addition, recent studies have implicated ILC2s in metabolism and metabolic diseases (Molofsky, Nussbaum et al. 2013, Hams, Locksley et al. 2013, Miller, Asquith et al. 2010, Nussbaum, Van Dyken et al. 2013).

1.3.1.3 *Group 3 ILCs (ILC3s and LTi cells)*

Group 3 ILCs is probably the most diverse category of ILCs (both in human and mouse) and includes LTi cells and ILC3s (Spits, Artis et al. 2013). Murine ILC3s can be further divided into CCR6 expressing ILC3s and $CCR6^-$ ILC3s. The later ($CCR6^-$ ILC3s) includes two distinct ILC3 subpopulations that can be distinguished by their expression of natural cytotoxicity receptors (NCRs) or the lack thereof into NCR^+ ILC3s and NCR^- ILC3s, respectively, whereas CCR6 expressing ILC3s are essentially the $CD4^+$ as well as $CD4^-$ LTi cells. In humans, the majority of ILC3s expresses CCR6, which can be further divided into two populations depending on whether or not they express the NCR NKp44 (Cella, Fuchs et al. 2009, Cupedo, Crellin et al. 2009, Satoh-Takayama, Vosshenrich et al. 2008b). Depending on the environmental stimulus, ILC3s can produce IL-17A, IL-17F, IL-22, TNFα and granulocyte macrophage colony-stimulating factor (GM-CSF) (Cella, Fuchs et al. 2009, Cupedo, Crellin et al. 2009, Satoh-Takayama, Vosshenrich et al. 2008b, Sonnenberg, Monticelli et al. 2011, Buonocore, Ahern et al. 2010), while they respond to IL-1β and IL-23 (Cella, Otero et al. 2010, Mortha, Chudnovskiy et al. 2014). In particular, LTi cells can produce IL-17A and IL-22 (Takatori, Kanno et al. 2009), as can do the NCR^- ILC3s (Satoh-Takayama, Vosshenrich et al. 2008b, Sonnenberg, Monticelli et al. 2011), whereas NCR^+ ILC3s produce only IL-22 (Spits, Di Santo 2011, Cella, Fuchs et al. 2009, Satoh-Takayama, Lesjean-Pottier et al. 2010, Vonarbourg, Mortha et al. 2010b).

Similarly to Th17 cells, all ILC3s require for their development the transcriptional factor RORγt (Eberl, Marmon et al. 2004, Satoh-Takayama, Vosshenrich et al. 2008a, Sun, Unutmaz et al. 2000), whereas their survival and effector functions depend on the transcriptional factor aryl hydrocarbon receptor (AHR) (Kiss,

Vonarbourg et al. 2011, Lee, Cella et al. 2011). Notably, recent evidence point to ILC3 plasticity and to T-bet as another transcriptional factor that can influence ILC3 fate (Klose, Flach et al. 2014, Klose, Kiss et al. 2013, Rankin, Groom et al. 2013). In particular, in the presence of cytokines such as IL-12 and IL-18, ILC3s can become IFNγ producing ILC1s by down-regulating RORγt whilst up-regulating T-bet (Vonarbourg, Mortha et al. 2010b, Klose, Kiss et al. 2013), a phenomenon that has also been observed in humans (Cella, Otero et al. 2010, Bernink, Peters et al. 2013a).

So far several studies have described a role for ILC3s in antibacterial immunity, chronic inflammation and tissue repair (Cella, Fuchs et al. 2009, Cupedo, Crellin et al. 2009, Satoh-Takayama, Vosshenrich et al. 2008b, Sonnenberg, Monticelli et al. 2011, Buonocore, Ahern et al. 2010). In the lung, ILC3s appear to provide protection against foreign pathogens such as the bacteria *Streptocucus pneumoniae* (Van Maele, Carnoy et al. 2014) and *Mycobacterium tuberculosis* (Pitt, Stavropoulos et al. 2012), as well as against the fungus *Candida albicans* (Gladiator, Wangler et al. 2013). ILC3s have also been associated with the inflammatory skin disease psoriasis vulgaris (Pantelyushin, Haak et al. 2012). NCR^+ ILC3s are present in the blood and skin of patients with psoriasis but absent in healthy individuals whose blood and skin contained only NCR^- ILC3s instead (Villanova, Flutter et al. 2014). A potential role for ILC3s has also been described in multiple sclerosis (Perry, Han et al. 2012), although whether they are protective or pathogenic remains unclear. Moreover, emerging data point to an important role for ILC3s in cancer (Shields, Kourtis et al. 2010), and in particular on their involvement in promoting colorectal cancer (Langowski, Zhang et al. 2006, Chan, Jain et al. 2014, Huber, Gagliani et al. 2012a, Kirchberger, Royston et al. 2013).

1.3.2 ILC3s in IBD

Accumulating evidence also suggests group 3 ILCs as key mediators of intestinal inflammation. ILC3s that develop and accumulate to the gastrointestinal tract and gut-associated lymphoid tissues in a microbiota-independent manner (Monticelli, Sonnenberg et al. 2011a, Lee, Cella et al. 2011, Sawa, Cherrier et al. 2010, Sonnenberg, Monticelli et al. 2012), secure intestinal homeostasis by regulating host-microbiota interactions. Sonnenberg *et al* in a land mark study showed that ILC3s have an important role in anatomically containing the commensal flora (Sonnenberg,

Monticelli et al. 2012). Loss of ILC3s in the intestine resulted in lower levels of IL-22 and reduced production of antimicrobial peptides by intestinal epithelial cells leading to peripheral dissemination of *Alcaligenes* bacteria and systemic inflammation (Sonnenberg, Monticelli et al. 2012). Notably, IL-22 administration was able to prevent systemic inflammation in this setting (Sonnenberg, Monticelli et al. 2012). Besides helping containing commensal bacteria within their natural environment (lumen), ILC3s promote intestinal homeostasis by regulating host's adaptive immune responses against its microbiota. Hepworth *et al* provided the first evidence of ILCs directly regulating adaptive immunity by showing that loss of RORγt expressing ILCs (group 3 ILCs) resulted in enhanced antigen-specific Th17 responses against commensal bacteria (Hepworth, Monticelli et al. 2013). Interestingly, RORγt$^+$ ILC functions were independent of the IBD relevant cytokines IL-17A, IL-22 and IL-23 (Hepworth, Monticelli et al. 2013). Instead, it was shown that ILC3s express MHC class II and therefore can process and present antigen to CD4$^+$ T cells, hence limiting pathological responses to commensal bacteria (Hepworth, Monticelli et al. 2013). Supporting the antigen-presenting capacity of intestinal ILC3s, an independent study showed that splenic ILC3s also express MHC class II, and co-stimulatory molecules and can prime T cells *in vitro* (von Burg, Chappaz et al. 2014). Shedding more light into ILC3-T cell interactions at the intestinal barrier, Korn *et al* showed that loss of CD4 T cells leads to increased ILC numbers in the intestinal lamina propria, as well as high production of IL-22 and AMPs (Korn, Thomas et al. 2014), further supporting the cross talk of ILC3s with T cells. In the gut, ILC3s also appear to interact with B cells (the other major cell type of adaptive immunity), as well as several innate immune cells such as macrophages and DCs in order to maintain intestinal homeostasis. Kruglov *et al* showed that RORγt expressing ILCs regulate both T cell dependent and independent IgA induction by production of soluble lymphotoxin α (sLTα3) and membrane-bound lymphotoxin β (LTα1β2), respectively (Kruglov, Grivennikov et al. 2013). Moreover, granulocyte-macrophage colony-stimulating factor (GM-CSF) producing ILC3s were shown to affect phagocyte functions resulting in decreased regulatory T cell numbers, and therefore to impaired oral tolerance (Mortha, Chudnovskiy et al. 2014). Taken all together, these studies strongly suggest that ILC3s have a crucial role in promoting and maintaining intestinal homeostasis.

Taking these findings a step further, someone could hypothesize that ILC3s may also have an important role during intestinal inflammation that could subsequently lead to the development of IBD. Indeed, several independent studies have shown evidence implicating ILC3s with intestinal inflammation and IBD pathogenesis. Satoh-Takayama *et al* showed that loss of NKp46^{+} RORγt expressing, IL-22 producing innate immune cells was associated with increased susceptibility to *Citrobacter rodentium* colitis (Satoh-Takayama, Vosshenrich et al. 2008b), implicating for the first time what were later named NCR^{+} ILC3s with intestinal inflammation. Other independent groups have also shown that IL-22 producing RORγt^{+} ILC3s exhibit protective functions in different murine models of IBD (Zheng, Valdez et al. 2008, Sawa, Lochner et al. 2011, Cox, Kljavin et al. 2012). On the other hand, Buonocore *et al* showed that RORγt expressing innate immune cells promote *Helicobacter hepaticus* induced, innate driven colitis via IL-23-mediated IL-17A and IFNγ production (Buonocore, Ahern et al. 2010), directly implicating ILC3s in the development of IBD. In accordance with that notion, IL-23 responsive ILC3s are increased in the inflamed intestine of CD, but not UC patients, compared to healthy individuals (Geremia, Arancibia-Carcamo et al. 2011). Complementing these studies, previous work in our lab by Powell *et al* have also identified ILC3s as key mediators of intestinal inflammation in mouse models of experimental colitis as well as in patients with IBD (Powell, Walker et al. 2012, Powell, Lo et al. 2015).

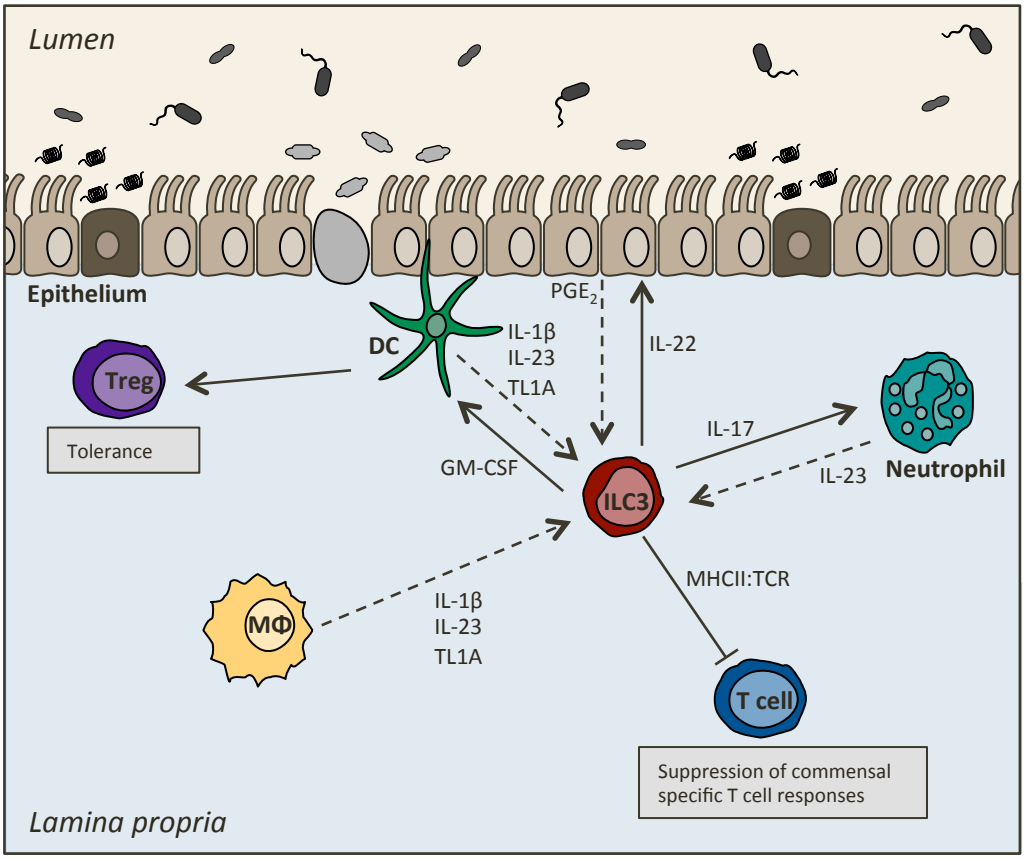

Figure 3: ILC3s & gut homeostasis. ILC3s are enriched at mucosal surfaces, and in particular in the lamina propria of the intestines. There, mononuclear phagocytes such as macrophages and DCs activate ILC3s via IL-1β, IL-23 and TL1A production. In turn, ILC3s secrete high amounts of IL-17A, IL-22 and GM-CSF to mediate their functions. IL-22 that acts primarily on the epithelium, promotes epithelial integrity and proliferation, induces antimicrobial peptide production by Paneth cells and mucin secretion by Goblet cells. IL-17A, which is also know for promoting epithelial integrity induces neutrophil recruitment. ILC3s regulate gut homeostasis by inducing oral tolerance through GM-CSF production, and by inhibiting antigen-specific T cell responses against commensal bacteria via MHC II:TCR interactions.

1.4 Interleukin 22

Growing evidence suggest interleukin 22 (IL-22), one of the signature ILC3 cytokines, as a key effector cytokine in the gastrointestinal track. In the gut, IL-22 has been shown to promote host's defence against pathogens, tissue regeneration, and to secure and maintain the integrity of the intestinal epithelial barrier. IL-22 was firstly identified by Dumoutier *et al* in IL-9 treated lymphoma cells as a T cell inducible factor related to IL-10 superfamily, hence originally named IL-10 related T cell derived inducible factor (IL-TIF) (Dumoutier, Louahed et al. 2000). Most cells of the innate and adaptive immune system are able to produce IL-22 including macrophages, DCs, neutrophils, mast cells, ILCs, NKT cells, conventional as well as γδ T cells (Cella, Fuchs et al. 2009, Cella, Otero et al. 2010, Lee, Cella et al. 2011, Korn, Thomas et al. 2014, Colonna 2009, Hanash, Dudakov et al. 2012, Lee, Yang et al. 2015, Mann, Bernardo et al. 2014, Sonnenberg, Fouser et al. 2011, Zindl, Lai et al. 2013), while fibroblasts provide a non-haematopoietic cellular source of IL-22 (Ikeuchi, Kuroiwa et al. 2005). In contrast to all other cytokines, IL-22 is the only cytokine that mediate her functions by acting almost exclusively on non-haematopoietic cells such as epithelial and stromal cells (Wolk, Kunz et al. 2004). In general, IL-23 and IL-1β are the main known inducers of IL-22 production (Kastelein, Hunter et al. 2007, Lee, Kumagai et al. 2013), whereas TGFβ and IL-25 appear to be negative regulators of IL-22 (Weiss, Wolk et al. 2004, Wolk, Witte et al. 2007, Zheng, Danilenko et al. 2007).

1.4.1 IL-22 signaling pathway

The IL-22 receptor (IL-22R) is a cell surface receptor comprising a heterodimer complex of IL-22R1, which is unique for IL-22 and IL-10R2 that is shared with the receptor for IL-10 (Li, Tomkinson et al. 2004). At first, IL-22 binds to IL-22R1 subunit causing a transformation that allows subsequently binding to the IL-10R2 subunit initiating the downstream signaling (Li, Tomkinson et al. 2004). Similarly to IL-10 signaling pathway, following IL-22 binding to its receptor, Janus kinase (JAK) and tyrosine kinase 2 (Tyk2) are activated and trigger the phosphorylation of signal transducer and activator of transcription 3 (STAT3) (Lejeune, Dumoutier et al. 2002, Naher, Kiyoshima et al. 2012, Jones, Logsdon et al. 2008). However, IL-22 can also

activated other kinases including p38, MEK/ERK and JNK, as well as trigger STAT1 and STAT5 phosphorylation (Lejeune, Dumoutier et al. 2002, Jones, Logsdon et al. 2008).

IL-22 also binds with high affinity to a soluble IL-22 receptor known as IL-22BP (Ciccia, Guggino et al. 2015). IL-22BP forms stable complexes with IL-22, essentially blocking it's binding to IL-22R1 and its signaling activation (de Moura, Watanabe et al. 2009). IL-22BP can be found in different tissues such as placenta, lung, skin and the gastrointestinal tract and is constitutively produced by colonic DCs (Huber, Gagliani et al. 2012b). IL-22BP is also expressed by macrophages, eosinophils, as well as epithelial cells, and has already been linked with inflammatory conditions (Weiss, Wolk et al. 2004, Dumoutier, Lejeune et al. 2001, Wei, Ho et al. 2003, Martin, Beriou et al. 2016, Yang, Gao et al. 2014).

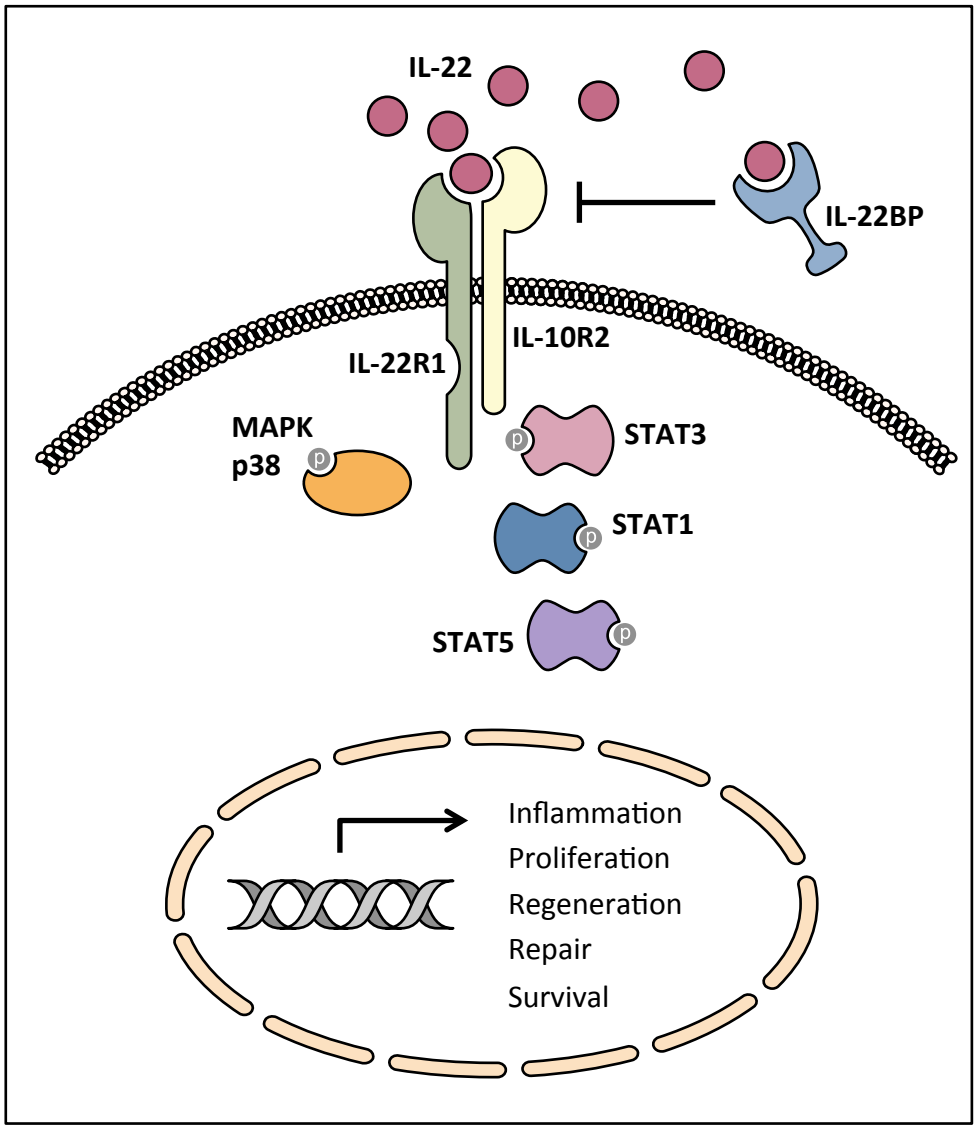

Figure 4: IL-22 signaling pathway. IL-22 binds to a heterodimer recepteror found in the cell membrane of several non-hematopoietic cells. Initially, a monomer of IL-22 binds to IL-22R1 causing a structural change that allows the complex to subsequently bind to IL-10R2 triggering the IL-22 signaling cascade. Upon activation, tyrosine and serine of STAT3 are phosphorylated through Jak1 and Tyk2 activation, although IL-22 can also activate kinase p38, MEK/ERK and JNK leading to STAT1 and STAT5 phosphorylation. In addition, IL-22 can bind with high affinity to a soluble receptor IL-22BP negatively regulating its actions. Ultimately, IL-22 signaling triggers the expression of multiple genes involved in cell proliferation and survival, tissue regeneration and repair, as well as inflammation.

1.4.2 Biologic effects of IL-22

In general, IL-22 has different functions depending on the target tissue. At barrier surfaces such as skin, lungs and the gastrointestinal tract, IL-22 is well known for its role in host defence against pathogens, whereas in other organs such as liver and pancreas IL-22 promotes cell survival and proliferation. In a mouse model of pancreatitis, IL-22 deficiency was associated with increased tissue damage and fibrosis (Feng, Park et al. 2012). In the liver, following hepatitis infection or tissue resection, IL-22 has been shown to promote tissue regeneration (Radaeva, Sun et al. 2004, Ki, Park et al. 2010, Brand, Dambacher et al. 2007). In the gut, IL-22 that is mainly produced by ILC3s seems to have a protective role against intestinal infections (Zheng, Valdez et al. 2008, Monticelli, Sonnenberg et al. 2011b), while its inhibition in murine models of IBD is associated with increased inflammation (Zenewicz, Yancopoulos et al. 2008, Sugimoto, Ogawa et al. 2008). Similarly to its actions in the gastrointestinal tract, several independent studies have shown a protective role for IL-22 in the lung, where IL-22 deficiency is associated with increased bacterial invasion (Aujla, Chan et al. 2008, Dhiman, Venkatasubramanian et al. 2014). However, despite its well-documented beneficial effects, IL-22 appears to be detrimental in other pathological conditions such as psoriasis (Wolk, Witte et al. 2009, Wolk, Haugen et al. 2009) and cancer. Increased IL-22 levels are associated with pathology in several types of cancer including lung, liver, and pancreatic cancers (Di Lullo, Marcatti et al. 2015, Nardinocchi, Sonego et al. 2015). In colorectal cancer, elevated IL-22 production leads to greater tumour burden and reduced survival (Huang, Cao et al. 2015, Koltsova, Grivennikov 2014, Kryczek, Lin et al. 2014). In conclusion, IL-22 seems to have both beneficial and detrimental effects depending on the tissue and/or the environmental context. Further investigation is needed to identify how these pathways can be regulated and/or exploited to treat complicated inflammatory conditions such as IBD and cancer.

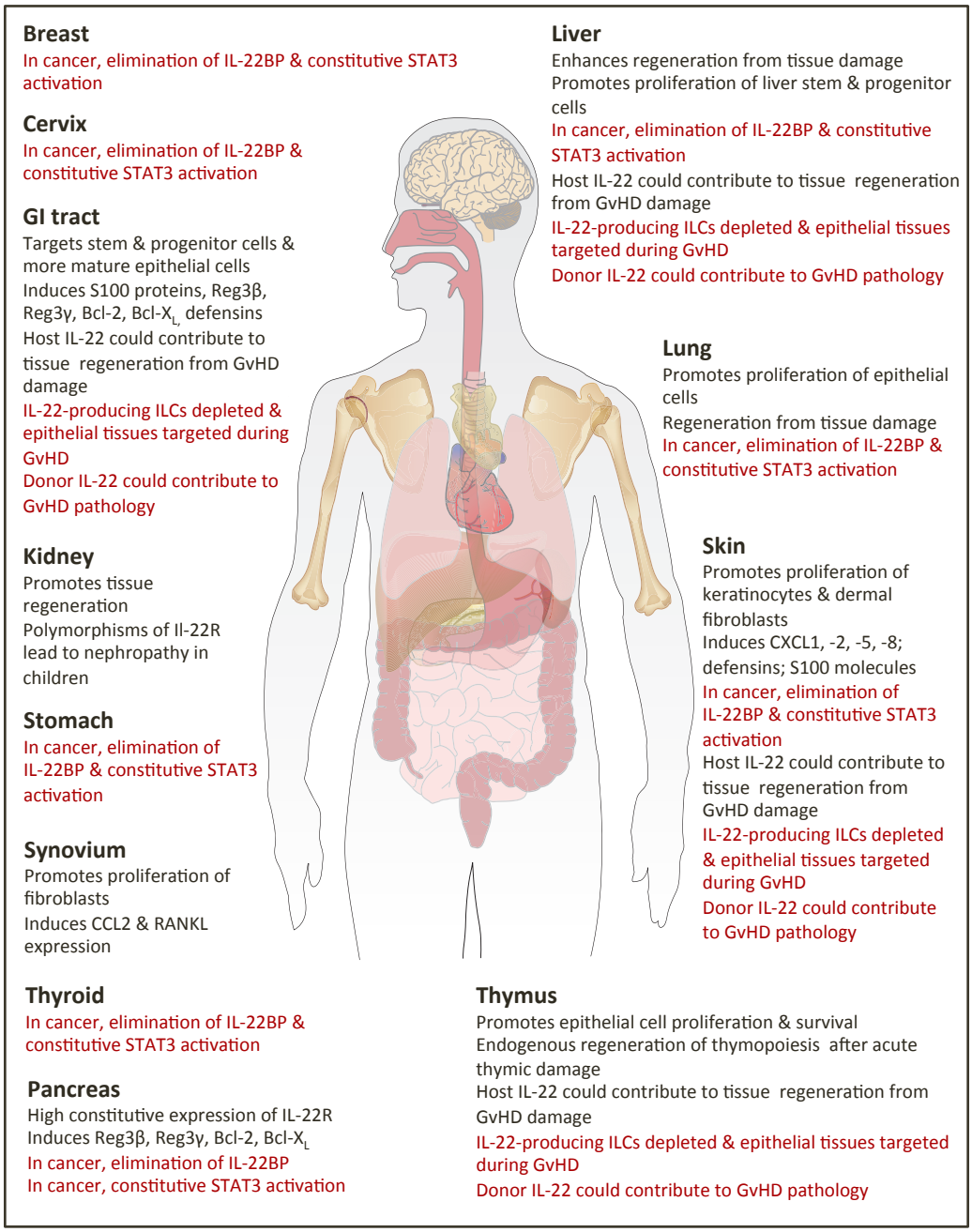

Figure 5: Beneficial (in black) & detrimental (in red) actions of IL-22 in the tissue. (Adapted from Dudakov, Hanash et al. 2015). IL-22 acts on epithelial cells and some fibroblasts in several different tissues. In the kidneys, liver, lung, skin, synovium, and thymus it promotes proliferation, tissue regeneration and wound healing, while in barrier surfaces including the lungs, intestines and the skin, IL-22 induces antimicrobial peptide production and secretion of defensins. However, IL-22 has also been implicated in several types of cancer such as breast, cervical, colorectal, pancreatic, liver, skin and thyroid cancer, as well as in other inflammatory conditions like GvHD.

Aims and hypothesis

Hypothesis

Several independent studies including previous work in this lab have implicated ILC3s with IBD pathogenesis. However, the majority of those studies focus on the role of the NCR^+ subset of group 3 ILCs, and their effects in the small intestine leaving the remaining ILC3 populations and their role in colonic inflammation somewhat understudied. Therefore, this thesis tests the hypothesis that NCR^- ILC3s are also important drivers of colonic inflammation and their effector pathways could also be explored for the generation of novel IBD therapies.

Aims

This thesis aims to identify how NCR^- ILC3s regulate colonic inflammation by:

1. Defining the transcriptional changes on these cells during colonic inflammation using mouse models of experimental IBD
2. Identifying their molecular mediators and cellular targets
3. Mapping the signaling pathway(s) involved revealing potentially new therapeutic targets

Chapter 2

Materials & Methods

2.1 Mice and animal husbandry

Balb/c *Tbx21*$^{-/-}$*Rag2*$^{-/-}$ double KO (TRUC) mice have been described previously (Powell et al., 2015; Powell et al., 2012). *Tbx21*$^{-/-}$*Rag2*$^{-/-}$*Il22*$^{-/-}$ (TRUC*Il22*$^{-/-}$) triple KO mice were generated by backcrossing Balb/c *Tbx21*$^{-/-}$*Rag2*$^{-/-}$ double KO (TRUC) mice with Balb/c *Il22*$^{-/-}$ mice that were provided by Pfizer. *Lgr5*-eGFP reporter mice were provided by Professor Fiona Watt, King's College London. *RORc*-eGFP reporter mice were provided by Professor Gerard Eberl, Institute Pasteur, Paris and have been described previously (Lochner et al., 2008). *RORc*-eGFP reporter mice were backcrossed with *Rag1*$^{-/-}$ mice to generate *Rag1*$^{-/-}$*RORc*-eGFP mice. WT C57Bl/6 mice were purchased from Charles River Laboratories. *Il10*$^{-/-}$ mice were provided by Professor Werner Muller, Faculty of Life Sciences, University of Manchester. Mice were housed in specific pathogen free (SPF) conditions. All mice were handled according to local (KCL) and national guidelines, and all our experimental protocols were reviewed and approved by our local ethics review committee. All animal experiments were conducted in accredited facilities in accordance with the UK Animals (Scientific Procedures) Act 1986 (Home Office license number PPL 70/7869).

2.2 Genotyping

Genomic DNA was extracted by digesting ear or tail biopsies in 250µl of lysis buffer (5mM EDTA, 100mM Tris-HCl pH 8.5, 0.2% SDS, 200mM NaCl, 1mg/ml proteinase K) at 56^{o}C o/n. Inactivation of proteinase K was achieved by incubating digested samples at 75^{o}C for 15min. Digested samples containing the genomic DNA were diluted 1:3 in nuclease-free H_2O and were used further for genotypic analysis. Each PCR reaction consisted of 1µl of genomic DNA, 12.5µl Mango Mix 2x

(Bioline Ltd., London, UK), 9.5µl nuclease-free H_2O and 1µl of each PCR primer (forward and reverse). 12µl of each PCR product were loaded on 1.5% or 2% agarose gel premixed with GelRed Nucleic Acid Gel Stain (Biotium) to visualise DNA bands following UV illumination (Unipro Gold). All primers and programs used for genotyping purposes are listed in tables 2 and 3, respectively.

Table 2: List of primers used for genotyping

Target gene	Primer	Sequence	Source
Rag1	olMR1746	GAGGTTCCGCTACGACTCTG	IDT
	olMR3104	CCGGACAAGTTTTTCATCGT	
	olMR8162	TGGATGTGGAATGTGTGCGAG	
Rag2	RagA	GGGAGGACACTCACTTGCCAGTA	IDT
	RagB	AGTCAGGAGTCTCCATCTCACTGA	
	RagNeo	CGGCCGGAGAACCTGCGTGCAA	
Tbx21	olMR1717	GCGCGAAGGGGCCACCAAAGAACGGAG	IDT
	olMR1718	GACTGAAGCCCCGACCCCCACTCCTAAG	
	olMR1719	TGGGCATACAGGAGGCAGCAACAAATA	
Il22	oIMR4090	GCCTCTCCCATCACAAGCAGAGACAC	IDT
	oIMR4091	GGCTGCTGGAAGTTGGACACCTCAAG	
	oIMR4092	GATACAGGTGCAGCTAAGCGAG	
***RORc*-eGFP**	3	CCCCCTGCCCAGAAACACT	IDT
	4B	GGATGCCCCCATTCACTTACTTCT	

Table 3: List of PCR programs used for genotyping

Target gene	Temperature	Time	No of cycles	Product size
Il22	94°C	10min	1	
	94°C	30sec		**WT: 380bp**
	57°C	30sec		
	72°C	1min	35	
	72°C	2min	1	**KO: 400bp**
	10°C	Forever		
Rag1	95°C	5min	1	
	94°C	30sec		**WT: 474bp**
	62°C	45sec		
	72°C	1min	35	
	72°C	10min	1	**KO: 530bp**
	4°C	Forever		
Rag2	95°C	5min	1	
	94°C	30sec		**WT: 300bp**
	62°C	45sec		
	72°C	1min	35	
	72°C	10min	1	**KO: 400bp**
	4°C	Forever		
***RORc*-eGFP**	95°C	10min	1	
	94°C	60sec		**WT: 250bp**
	55°C	60sec		
	72°C	60sec	35	
	72°C	2min	1	**KI: 1000bp**

	4°C	Forever		
Tbx21	94°C	3min	1	
	94°C	30sec		**WT: 263bp**
	60°C	60sec		
	72°C	30sec	35	
	72°C	2min	1	**KO: 450bp**
	10°C	Forever		

2.3 Animal models of experimental colitis

2.3.1 DSS

3% DSS (MW 36,00-50,000, MP Biomedicals, LLC) was administered orally in drinking water for 5 or 6 days to C57Bl6 mice and animals were culled at day 7 or 8 respectively. Food consumption and water intake were measured (and recorded) daily throughout the period of DSS administration to ensure that any differences due to variations on DSS dosage are accounted for. Mice were monitored daily and scored for weight loss, rectal bleeding and faeces consistency. Disease activity index was calculated as the sum of the above scores divided by 3 according to the table below {{478 Cooper,H.S. 1993;}}. All animals were daily thoroughly observed for general signs of distress or adverse symptoms and any mice presented with these features were humanely culled on welfare grounds.

Table 4: Scoring of disease activity index (DAI). (Adapted from Cooper HS et al., 1993).

Score	Weight loss	Stool consistency	Rectal bleeding
0	None	Normal	Normal
1	1 - 5 %		
2	5 - 10 %	Loose stools	
3	10 - 20 %		
4	> 20 %	Diarrhoea	Gross bleeding

2.3.2 DNBS

200µl of 3mg DNBS (Sigma-Aldrich) resolved in 50% EtOH were administered rectally while mice were under isoflurane anaesthesia. Mice were monitored daily for weight loss, general signs of distress and adverse disease symptoms. Any mice presented with these features were humanely culled on welfare grounds; otherwise mice were culled 3 days post administration for further analysis. DNBS

administration was performed by Dr Nicholas Powell with the kind help of Stuart Newman (BSU staff).

2.3.3 TCT

0.5 or 2 x10^6 naïve $CD4^+$ T cells (defined as live $CD4^+CD25^-CD44^{lo}CD62L^{hi}$ cells) were FACS sorted from spleens of 8-week-old C57Bl6 WT female or male donor mice, and injected (in 200µl of sterile PBS) intraperitoneally into 8-10 week old C57Bl6 *Rag1*$^{-/-}$ recipients. Purity checks were performed at the end of every sort and cells were always found more than 97% pure. Recipient mice were monitored twice per week for weight loss, general signs of distress and adverse symptoms. Any mice presented with these features were humanely culled on welfare grounds; otherwise mice were culled 4 to 6 weeks post adoptive transfer for further analysis.

2.3.4 *Il10*$^{-/-}$

Il10$^{-/-}$ mice were introduced to HT and TRUC microbiota by oral gavage, which was kindly performed by Dr Emilie Stolarzcyk. Mice were monitored twice per week for weight loss, general signs of distress and adverse symptoms. Any mice presented with these features were humanely culled on welfare grounds; otherwise mice were culled 4 weeks post gavage for further analysis.

2.3.5 TRUC

TRUC mice are descendants of the TRUC colony generated at Harvard {{201 Garrett,W.S. 2007;}}, while TRnUC mice were generated at KCL by backcrossing commercially available Balb/c *Tbx21*$^{-/-}$ *mice* with *Balb/c Rag2*$^{-/-}$ mice as described previously {{122 Powell,N. 2012;}}. TRUC*Il22*$^{-/-}$ mice were generated at KCL by backcrossing TRnUC mice with *Il22*$^{-/-}$ mice that were obtained from Pfizer. Due to the transmitability of TRUC disease TRUC mice were bred and housed at a different isolator at all times.

2.4 *in vivo* treatment of mice

Neutralizing anti-IL-22 mAb (clone IL22-01) and recombinant IL-22 (rIL-22) were developed and provided by Pfizer. 200µg of IL22-01 (per mouse) were administered *ip*. every 3 to 4 days. 100µg of rIL-22 (per mouse) were administered *ip*. at days 0, 4,

8 and 12, while mice were culled at day 14. 150μg of anti-CXCL1 (clone 124014, R&D Systems) were administered *ip* every 3 to 4 days. Anti-CXCR2 (clone 242216, R&D Systems) was administered *ip*. at a dose of 100μg per mouse at days 0, 3, 7, 10 and 14, while mice were culled at day 15. Tunicamycin (1μg per mouse) was dissolved in 0.1% DMSO and was administered rectally to TRUC*Il22*$^{-/-}$ mice at day 0 and 5. Control mice were administered 0.1% DMSO alone. 4-phenylbutyric acid (4-PBA) (Sigma Aldrich) was administered orally (in drinking water) to TRUC mice at a final concentration of 20mM for 6 weeks. *Ip*. injections were kindly performed by Dr Nicholas Powell and Dr Luke Roberts. Tunicamycin (Sigma Aldrich) administration was performed by Dr Nicholas Powell with the kind help of Stuart Newman (BSU staff) for the administration of inhaled anaesthesia.

2.5 Tissue collection and single cell preparation

Mice were euthanized by either cervical dislocation or by a rising concentration of carbon dioxide gas, and then dissected in a laminar flow cabinet under aseptic conditions. Once dissected, spleens and MLNs were placed into ice-cold complete cell culture media, while colons were first flashed with ice-cold PBS to remove faeces and then transferred into fresh ice-cold PBS. Single cell suspensions from spleen and MLN were prepared by mechanical disruption of the tissue using nylon mesh. To remove any red blood cells, cells isolated from spleen (but not MLN) were resuspended in 2ml ACK lysis buffer (0.15M NH_4Cl, 1mM $KHCO_3$, 0.1mM Na_2EDTA, pH=7.2-7.4) and incubated at RT for 2min. All cell washes were performed using complete cell culture media or ice cold PBS and centrifugation at 1800rpm, 4^{o}C for 5min, unless stated otherwise. Cell counts were measured manually using Trypan Blue staining (1:2 dilution) to distinguish live from dead cells.

2.6 Isolation of colonic LP leukocytes (cLPMCs)

Colons were opened longitudinally, cleaned thoroughly with ice-cold PBS and cut into 1-2mm pieces and washed with 10ml 5mM EDTA, 1mM Hepes in HBSS (Gibco) in a shaking water bath (300rpm) at 37^{o}C for 20min. Tissue was then

vortexed vigorously for 10sec and passed through a 100μM cell strainer and collected in C-tubes (Miltenyi) in complete RPMI (Gibco) containing 10% fetal calf serum, 0.25mg/ml Collagenase D (Roche), 1.5mg/ml Dispase II (Roche) and 0.01μg/ml DNase (Roche) and put in a shaking water bath (300rpm) at 37^{o}C for 40min. Before and after the 40min incubation C-tubes were vigorously shaken for 30sec. Solutions were then passed through 100μM cell strainers and washed with ice-cold PBS. Cells were resuspended in 10ml of the 40% fraction of a 40:80 Percoll (GE Healthcare) gradient and carefully placed on top of 5ml of the 80% fraction in 15ml tubes. Percoll gradient separation was performed by 20min centrifugation at 2600rpm at room temperature without break. LP cells were collected from the interphase of the gradient and washed with ice-cold PBS. Cells were resuspended in 1ml PBS, counted and immediately used for further experiments.

2.7 Immunomagnetic based cell separation

Enrichment of CD4^{+} T cells from the spleen was achieved by positive selection using magnetic cell purification. Unfractionated single cells were firstly introduced to ACK lysis buffer to remove red blood cells (as described above) and then washed in ice-cold PBS by 10min centrifugation at 1800rpm, 4^{o}C. Cells were resuspended in MACS buffer (PBS, 2% FCS, 1mM EDTA) and incubated with anti-CD4 magnetic beads for 20min on ice according to manufacture's instructions. Cells were washed of excess beads with MACS buffer by 5min centrifugation at 1800rpm, 4^{o}C and resuspended in 500ul of MACS buffer before passing through MACS LS magnetic columns attached to a quadroMACS magnet. Columns were washed 3 times with 3ml MACS buffer before positively selected CD4^{+} T cells were eluted with 1ml PBS. Cells were washed with complete cell culture media and stored on ice pending further analysis. All reagents and equipment were supplied by MIltenyi Biotec Gmbh, Bergisch Gladbach, Germany.

2.8 Flow cytometry

2.8.1 Cell surface and intracellular/intranuclear stainings

Single cell suspensions from spleens, MLNs and colonic tissue were washed with ice cold PBS and centrifugation at 1800rpm, 4^{o}C for 5min prior to all staining. Cells were then resuspended in 200µl PBS containing Fc block (aCD32/CD16, eBioscience) at 1:100 dilution and incubated on ice for 10min. Antibodies against all surface markers were added at appropriate dilutions as well as LIVE/DEAD Fixable Dead Cell Stain (Invitrogen) used in 1:1000. Samples were mixed by vortex and incubated for another 20min on ice in the dark. After the incubation, cells were washed with ice cold PBS and centrifugation at 1800rpm, 4^{o}C for 5min and then fixed with 400µl of 4% PFA and incubated at RT for 15min in the dark. After fixation, cells were washed again with ice cold PSB, resuspended in 150-200µl PBS and stored at 4^{o}C in the dark awaiting sample acquisition. For intracellular/intranuclear staining, after the incubation with antibodies against cell surface markers, cells were washed and resuspended in 1ml Cytofix/Cytoperm (eBioscience) following o/n incubation at 4^{o}C to accomplish cell/nuclear permeability. Cells were then washed with freshly made Perm buffer (eBioscience), and stained with mAbs against intracellular/intranuclear markers diluted 1:200 in Perm buffer (containing Fc block at 1:200) for 45min on ice in the dark. After the ICC staining, cells were washed with perm buffer and resuspended in 150-200µl PBS and stored at 4^{o}C in the dark awaiting sample acquisition. All monoclonal antibodies, live/dead dyes and other reagents used for FACS in this thesis are list in the table below:

Table 5: List of mAb used for FACS

Antigen	Fluorochrome	Clone	Source
CD11b	FITC	M1/70	BD
CD127	APC	eBioRDR5	eBioscience
CD127	BUV737	SB/199	BD
CD25	APC	PC61.5	eBioscience
CD27	BV605	LG.3A10	BD
CD3	BUV395	145-2C11	BD
CD4	BV786	RM4-5	BD
CD4	FITC	GK1.5	eBioscience
CD44	PE	IM7	eBioscience
CD45	Pac. orange	MCD4530	Invitrogen
CD45	V500	30-F11	BD
CD62L	eFlour®450	MEL-14	Biolegend
epCAM	eFlour®450	G8.8	eBioscience
Gr-1	PE	RB6-8C5	eBioscience
Gr-1	APC-Cy7	RB6-8C5	eBioscience
iCOS	APC	C398.4A	eBioscience
IL-17R	APC	PAJ-17R	eBioscience
IL-22R	PE	496514	R&D
KLRG1	PerCP- eFlour®710	2F1	eBioscience
NKp46	PE-Cy7	29A1.4	eBioscience
RORγt	PE	B2D	eBioscience
RORγt	BV786	Q31-378	BD

2.8.2 Sample acquisition and analysis

All samples were acquired on a BD LSRFortessa™ (BD Biosciences) at the Biomedical Research Council (BRC) Flow Core (15th Floor, Tower Wing, Guy's Hospital). Data were analysed using FlowJo software (Treestar).

2.8.3 Cell sorting

To obtain a pure population of naïve $CD4^+$ T cells from the spleen, splenic single cell suspensions were first treated with ACK buffer for red blood cell lysis, enriched for $CD4^+$ cells using immunomagnetic based cell separation and then stained with mAbs against CD4, CD25, CD44 and CD62L and LIVE/DEAD Fixable Dead Cell Stain (Invitrogen, UK) as described above. Naïve $CD4^+$ T cells were defined as live $CD4^+CD25^-CD44^{lo}CD62L^{hi}$ cells. Purity checks were performed after every sort and purity was always found to be above 97%. To obtain pure populations of colonic ILC2s and NCR^- ILC3s, cLPMCs were stained with mAbs against CD45, CD90, CD127, KLRG1 and NKp46 and Live/Dead dye as described above. ILC2s and NCR^- ILC3s were defined as live $CD45^+CD90^+CD127^+KLRG1^+NKp46^-$ and $CD45^+CD90^+CD127^+KLRG1^-NKp46^-$ cells respectively unless stated otherwise. Intestinal epithelial stem cells were sorted from murine colonoids that were generated from *Lgr5*-eGFP reporter mice as single live $epCAM^+$(*Lgr5*)-$eGFP^+$ cells. Regulatory T cells (Tregs) were sorted from the spleen of FoxP3-eGFP reporter mice as live $CD4^+CD25^+$(FoxP3)-$eGFP^+$ cells. Tregs from spleens of WT and *Il10*$^{-/-}$ mice were sorted as live $CD4^+CD25^+CD62L^{hi}$ cells. All sorts were performed on BD Aria I, BD Aria II or BD Aria Fusion (BD Biosciences) at the BRC Flow Core (15th Floor, Tower Wing, Guy's Hospital) by experienced members of staff.

2.9 Cell cultures

FACS purified NCR^- ILC3s isolated from the colon of TRUC or TRUC*Il22*$^{-/-}$ mice were always cultured for 24h in complete RPMI (Gibco) containing 10% FCS in the presence or absence of 10ng/ml IL-23 and 10ng/ml IL-1β unless stated otherwise. For the co-culture experiments NCR^- ILC3s isolated from the colon of TRUC or TRUC*Il22*$^{-/-}$ mice were activated for 48h with 20ng/ml IL-2, 50ng/ml IL-7, 10ng/ml IL-23 and 10ng/ml IL-1β prior to being co-cultured with colonoids.

2.10 Colonoid cultures

All colonoids that were used for experiments described in this thesis were developed and maintained by our collaborator Dr Anastasia Tsakmaki. Mouse colonic crypts were isolated, cultured, and grown into organoids as described previously {{479 Sato,T. 2011;}}. Mouse crypts were cultured in growth medium containing advanced Dulbecco's modified Eagle's medium/F12, penicillin/streptomycin (100 units/mL), 10mM HEPES, 2mM Glutamax, supplements N2 (1x) and B27 (1x), 50ng/mL mouse epidermal growth factor (all obtained from Life Technologies), 1mM N-acetylcysteine (Sigma-Aldrich), 50% v/v Wnt3a conditioned medium, 10% v/v R-spondin-1 conditioned medium, 10% v/v Noggin conditioned medium and 3μM CHIR99021 (Cambridge Biosciences). Medium was changed every 2 days. Differentiation towards a mature epithelium was achieved by withdrawal of Wnt3a for 3 days. During the last 24h in differentiation medium colonoids were treated with 10ng/ml IL-22 or with 50ng/ml IL-17A. For the co-culture experiments, NCR^- ILC3s that were isolated from the colon of TRUC and TRUC*Il22*$^{-/-}$, FACS purified and activated as described above, were then resuspended in differentiation medium and co-cultured with colonoids at an ILC/crypt ratio of 25:1. After 24 hours ILCs were removed, colonoids were harvested from matrigel, washed with PBS and processed for RNA extraction and downstream analysis.

2.11 Cell lines

Mode K cell line was obtained from Dr Dominique Kaiserlian (INSERM) {{480 Vidal,K. 1993;}}. Cells were cultured in complete DMEM (Gibco) containing 10% FCS and were split 1:4 when confluent. Once confluent cells were lift up using 0.05% trypsin-EDTA (Gibco), spin down at 1800rpm for 5 min, and then cells were either frozen down in 1ml 90% FCS-10% DMSO or plated in 6-well plates at $2x10^6$/well in 3ml/well complete DMEM (Gibco) containing 10% FCS for further experiments. To measure CXCL1 and CXCL5 production followed by IL-22 treatment, Mode K cells were cultured with 10ng/ml IL-22 in the presence or absence of selective pathway inhibitors together with vehicle control (DMSO 0.1%). The inhibitors tested were NSC74859 85μM (STAT3 inhibitor, Tocris Bioscience),

SB203580 10μM (p38 inhibitor, Sigma-Aldrich), LY294002 10μM (PI3K inhibitor, Sigma-Aldrich), SP600125 10μM (JNK inhibitor, Sigma-Aldrich), BAY11-7082 2μM (NF-κB inhibitor, Tocris Bioscience), TC-S7006 5μM (MAP3K8 inhibitor, Tocris Bioscience) and PD98059 1μM (MEK1 inhibitor, Tocris Bioscience).

2.12 ELISA

Cytokine concentrations in supernatants (S/Ns) of either stimulated cell cultures or explant cultures were measured by ELISA. At the endpoint, S/Ns were harvested and stored at -20^{o}C pending further analysis. IL-22 and IL-17A ELISA kits were purchased from eBioscience and ELISAs were performed according to the manufacturer's protocols. Cytokine concentrations were determined within the linear phase of a standard curve made with known cytokine concentrations provided by the supplier.

2.13 RNA extraction

Whole tissue colonic fragments or FACS purified cells were lysed in 1ml TRIsure (Bioline) and stored at -80^{o}C pending further processing. Samples were left to thaw at RT and homogenized by vortex for 10sec. To extract the RNA, 200μl of chloroform were added to each sample followed by 10sec vortex and 15min incubation at RT. Samples were centrifuged at max speed for 15min at 4^{o}C and the clear S/N phase (containing the RNA) was transferred to new 1.5ml eppendorf tubes and then mixed with equal volume of isopropanol. Samples were vortex and then left at RT for 10min, followed by 8min centrifugation at max speed at 4^{o}C. RNA pellets were rinsed with 0.5ml of 75% EtOH and left to airdry at RT. Depending on pellet size; RNA was dissolved in 10-100μl of RNase/DNase free H_2O and stored at -80^{o}C awaiting further analysis.

2.14 cDNA synthesis (reverse transcription)

RNA samples were left to thaw on ice and the amount of RNA per sample was measured with NanoDrop. 11μl of RNA sample (always containing the same amount of RNA across all samples of the same experiment) that was always less that 4μg

RNA, were mixed with 1µl oligo dT and incubated at 65^{o}C for 5min. At the end of the incubation, RNA samples were mixed with 8µl of reverse transcription mix containing 4µl Buffer 5x, 1µl RNase Inhibitor (RI) at 20U/µl, 2µl dNTPs and 1µl Reverse Transcriptase (RT). Reverse transcription was then accomplished by incubating RNA samples at 42^{o}C for 1h followed by 65^{o}C for 5min and then 4^{o}C forever. cDNA samples (20µl) were stored at -20^{o}C until further use.

2.15 RT-qPCR

Total RNA was extracted from colonoids using RNeasy mini kit (Qiagen) and RNase-free DNase set (Qiagen) and reverse transcribed using a high-capacity cDNA reverse transcription kit (Life technologies) according to manufacturer's instructions. Quantitative PCR was performed using QuantiTect primers (Qiagen) and Quantitect SybrGreen MasterMix (Qiagen) on a LightCycler 480 (Roche). Sequences for mouse *sXbp1* splicing primers were obtained from Kaser *et al. {{432 Kaser,A. 2008;}}*. Samples were analysed in triplicates and relative expression of mRNAs was determined after normalisation against the housekeeping gene Beta-2-Microglobulin (B2M).

2.16 Microarrays and data analysis

2.16.1 Sample collection and preparation

ILC2s and NCR^{-} ILC3s were isolated from colon and sorted using a BD FACS Aria III cell sorter (BD Biosciences) following LIVE/DEAD Fixable Dead Cell Stain (Invitrogen) and labelled with antibodies conjugated to fluorochromes, CD45, CD90, CD127, KLRG1, and NKp46. RNA from sorted cells and from colonic tissue fragments (distal region) was extracted using TRIsure (Bioline) as described above. Contaminating DNA was removed with the RNase-Free DNase Set (Qiagen) according to the manufacturer's protocol. cDNA was synthetized using Ovation PicoSL WTA System V2 according to the manufacture's protocol (Nugen, USA) and labelled using Encore BiotinIL module according to the manufacture's protocol (Nugen, USA). RNA and cDNA quantity and quality were assessed using the Agilent RNA 6000 Nano Kit or Agilent RNA 6000 Pico Kit (depending on the amount of

RNA) according to the manufacture's protocol (Agilent Technologies, USA). Labelled cDNA were hybridised on a MouseWG-6 v2.0 Expression BeadChip (Illumina, USA).

2.17 Data analysis

All raw data [from experiments described in this thesis, as well as those obtained from the Gene Expression Omnibus (GEO) repository (Accession number GSE59071) {{254 Vanhove,W. 2015;}}] were analysed using Partek® Genomic Suite Software and GraphPad PRISM® version 7 software.

2.18 Histology

Whole colons or 1cm tissue fragments from the distal end (1cm away from the anus) were removed and fixed in 10% PFA (CellPath). Tissue processing, cutting as well as haematoxylin and eosin (H&E) staining of 5um sections (1-3 per sample) were performed at the Pathology Core of Blizard Institute (Barts and the London). All histology slides were blindly scored for colitis by Professor Tom T MacDonald (QMUL) based on signs of mononuclear infiltrate (0-3), polymorphonuclear infiltrate (0-3), epithelial hyperplasia (0-3) and epithelial injury (0-3). All histology pictures were acquired with Eclipse Ti-E Live Cell Imaging System 2 microscope at the Nikon Centre (KCL) using 40x magnitude. Data were analysed at the Nikon Centre (KCL) using NIS Elements AR software.

2.19 Statistical analysis

All graphs were generated and analysed using GraphPad Prism 7 software. Data represent mean or mean with SD unless stated otherwise. Statistical analysis was performed using non-parametric Mann-Whitney test or one-way ANOVA unless stated otherwise. Statistical significance was indicated using * for p values less than 0.05, ** for p values less than 0.01 and *** for p values less than 0.001 unless stated otherwise.

Chapter 3

Results: IL-22 producing NCR$^-$ ILC3s are an important ILC subset in colonic inflammation

ILCs are enriched at barrier surfaces and especially in the gut, where they act as effector cells enhancing host immunity against viruses, bacteria and parasites, whilst promoting wound healing and tissue regeneration. In particular, ILC3s are known to have an important role in host defence against bacterial infections (Satoh-Takayama, Vosshenrich et al. 2008, Sonnenberg, Fouser et al. 2011), which is at least partly dependent on IL-22 mediated induction of antimicrobial peptide responses (Zheng, Valdez et al. 2008). Group 3 ILCs are also involved in the anatomical containment of lymphoid tissue-resident commensal bacteria (Sonnenberg, Monticelli et al. 2012), as well as in the regulation of specific T cell responses against those bacteria (Hepworth, Monticelli et al. 2013). During the resolution stage of inflammation, ILC3s show involvement in tissue repair by promoting proliferation of Lgr5$^+$ stem cells (Lindemans, Calafiore et al. 2015).

On the other end of the spectrum, many studies have shown ILC3s to promote intestinal inflammation via IFNγ (Fuchs, Vermi et al. 2013, Buonocore, Ahern et al. 2010) and IL-17 production (Buonocore, Ahern et al. 2010, Powell, Walker et al. 2012). Moreover, cytokine producing ILCs are expanded in the gut of CD and UC patients, the two main forms of IBD (Geremia, Arancibia-Carcamo et al. 2011, Powell, Lo et al. 2015), whereas they are shown to have an important role in preclinical models of IBD (Buonocore, Ahern et al. 2010, Powell, Walker et al. 2012, Powell, Lo et al. 2015). Taken together, ILCs and/or their cytokine mediators may constitute new therapeutic strategies to treat IBD.

Despite the profound effector functions of group 3 ILCs in the intestinal barrier, most studies focus on NCR$^+$ ILC3s, while NCR$^-$ ILC3s and their functional role *in vivo* remains poorly understood. Furthermore, the majority of studies focus on the small intestine leaving the colonic ILC subsets and their functions under-investigated. By

investigating the role of NCR$^-$ ILC3s in colitis, this thesis aims to provide new insights into the role of NCR$^-$ ILC3s in colonic inflammation.

3.1 NCR$^-$ ILC3s are the predominant colonic ILC3 subset in health and disease

To begin to understand the role of NCR$^-$ ILC3s in colonic inflammation, the frequency of these cells in the colonic LP was investigated in steady state as well as in several models of experimental colitis. To do so, mononuclear cells were isolated from the colonic LP of *Rorc*-eGFP reported mice (Lochner, Peduto et al. 2008), stained with fluorochrome-conjugated antibodies against cell surface markers and analysed by flow cytometry. RORγt expressing ILC3s and NCR$^-$ ILC3s were identified using the gating strategy shown in Figure 6. In steady state, KLRG1$^+$ ILC2s and RORγt$^+$ ILC3s were the dominant ILC subsets in the colon (Figure 6). Within group 3 ILCs, NCR$^-$ ILC3s were the most common subset accounting for more than 70% of the ILC3 population (Figure 8). Similarly to healthy colon, after induction of colitis in *Rorc*-eGFP mice using DSS and DNBS as described in Figure 7, NCR$^-$ ILC3s were the main ILC3 subset in the colonic LP, although a slight decrease on the percentage of these cells was also observed (Figure 8). The frequency of NCR$^-$ ILC3s was then examined in the T cell transfer model of colitis (Powrie, Leach et al. 1993) by generating *Rag1*$^{-/-}$*Rorc*-eGFP mice, to which 2x10^6 million of naïve (CD25$^-$CD44^{lo}CD62L^{hi}) CD4$^+$ T cells were adoptively transferred *ip.* as described in Figure 7. Again, NCR$^-$ ILC3s were the most abundant ILC3 population in the colonic LP (Figure 8). Finally, to investigate the frequency of ILC3s in the *Il10*$^{-/-}$ model of chronic colitis, a different multi-colour Ab panel for flow cytometry was developed to allow use of the same gating strategy (Figure 6) for ILC3 identification but with intracellular staining for the transcriptional factor RORγt. NCR$^-$ ILC3s were yet again the most abundant ILC3 subset, although an expansion of these cells was also observed (Figure 8). Taken together these data suggest that in steady state as well as during intestinal inflammation the majority of ILC3s in the colonic LP is NCR$^-$ ILC3s, although it is not clear whether NCR$^-$ ILC3 numbers are actually affected upon intestinal inflammation.

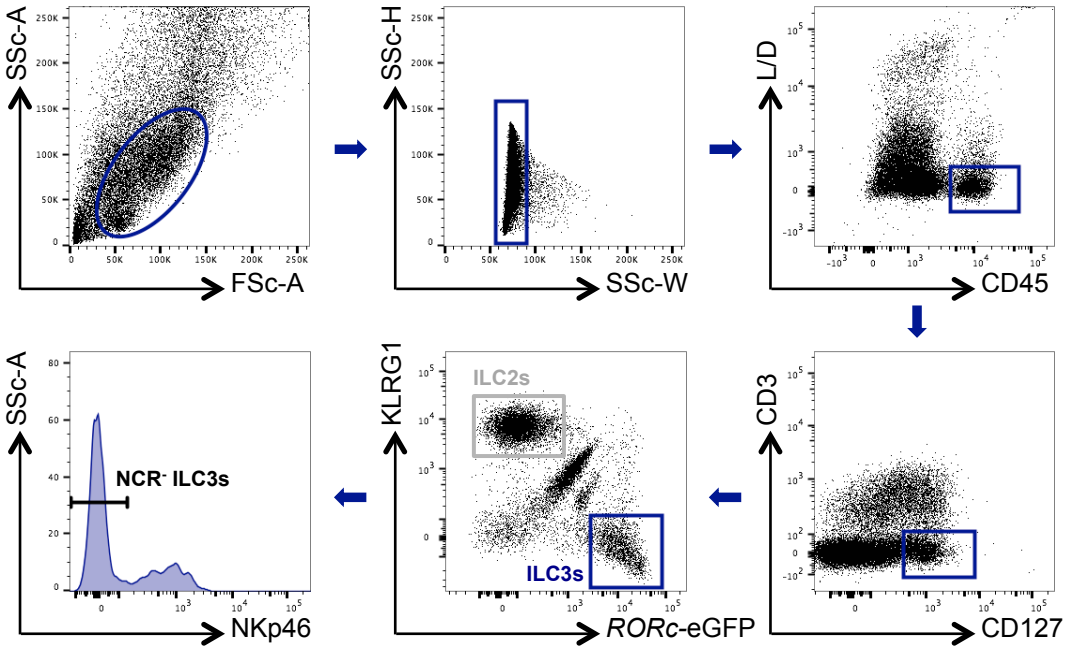

Figure 6: Gating strategy used to identify NCR$^-$ ILC3s in colonic LP. Flow plots representative of the gating strategy used to identify ILC2s and NCR$^-$ ILC3s in all experiments described in this thesis. In all experiments, where *RORc*-eGFP or *Rag1*$^{-/-}$*RORc*-eGFP mice where used, RORγt$^+$ ILC3s were identified based on GFP expression. In all other experiments, where mice didn't contain the *RORc*-eGFP transgene (*Il10*$^{-/-}$, *Rag2*-/-, TRUC mice) a fluorochrome-conjugated Ab that binds RORγt was used instead. Data were analysed using FlowJo software (Treestar).

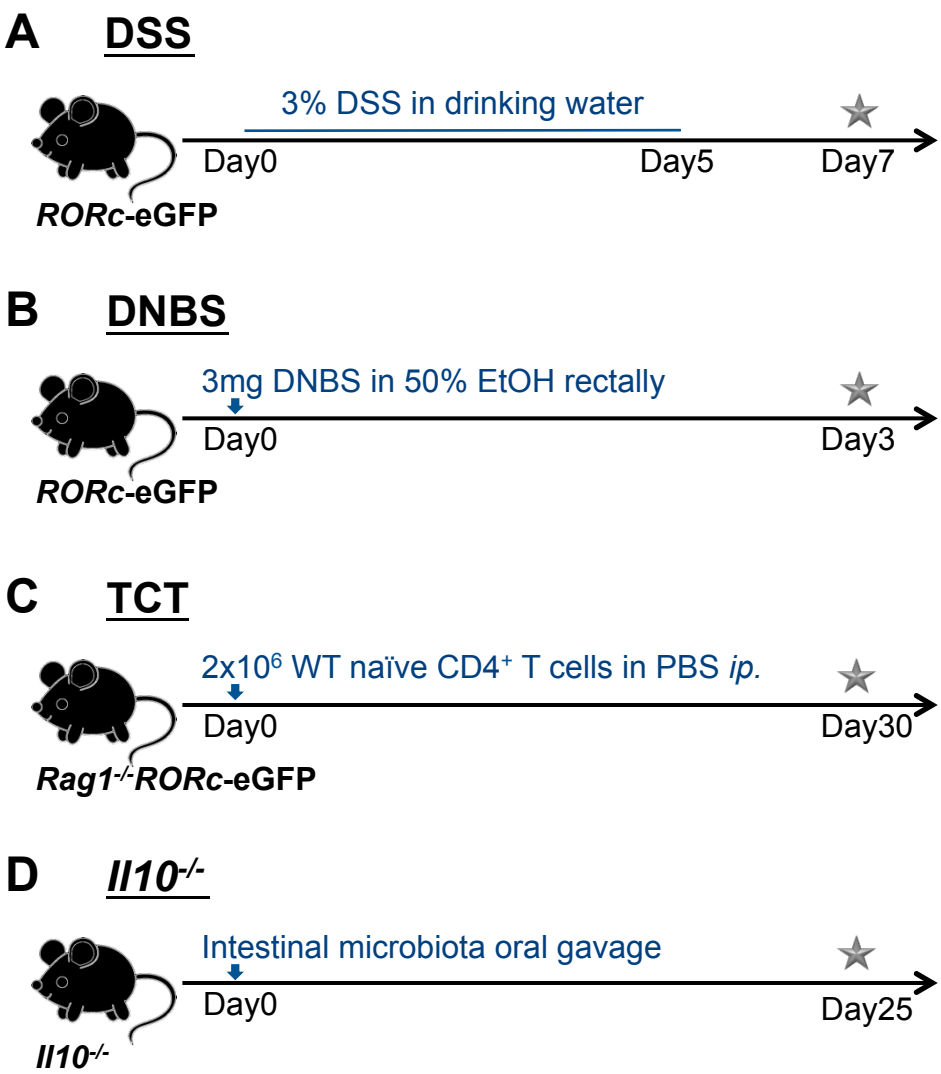

Figure 7: Protocols for induction of experimental colitis *in vivo*. A. 3% DSS in drinking water was administered to 8-12 week old *RORc*-eGFP mice for 5 days. Mice where switched to normal water at day 5 and culled at day 7 for further analysis. B. 3µg DNBS in 200µl 50% EtOH were administered rectally to 8-12 week old *RORc*-eGFP mice under isoflurane anaesthesia. Animals were culled 3 days later for further analysis. C. $2x10^6$ FACS purified naïve $CD4^+$ T cells from spleens of 8-week-old female WT mice were injected *ip.* to 8-12 week-old *Rag1*$^{-/-}$*RORc*-eGFP male and female recipients in 200µl sterile PBS at day 0. Mice were culled 30 days later for further analysis. D. 8-12 week old *Il10*$^{-/-}$ mice were gavaged orally with intestinal microbiota harvested from TRUC mice at day 0 and culled at day 25 for further analysis.

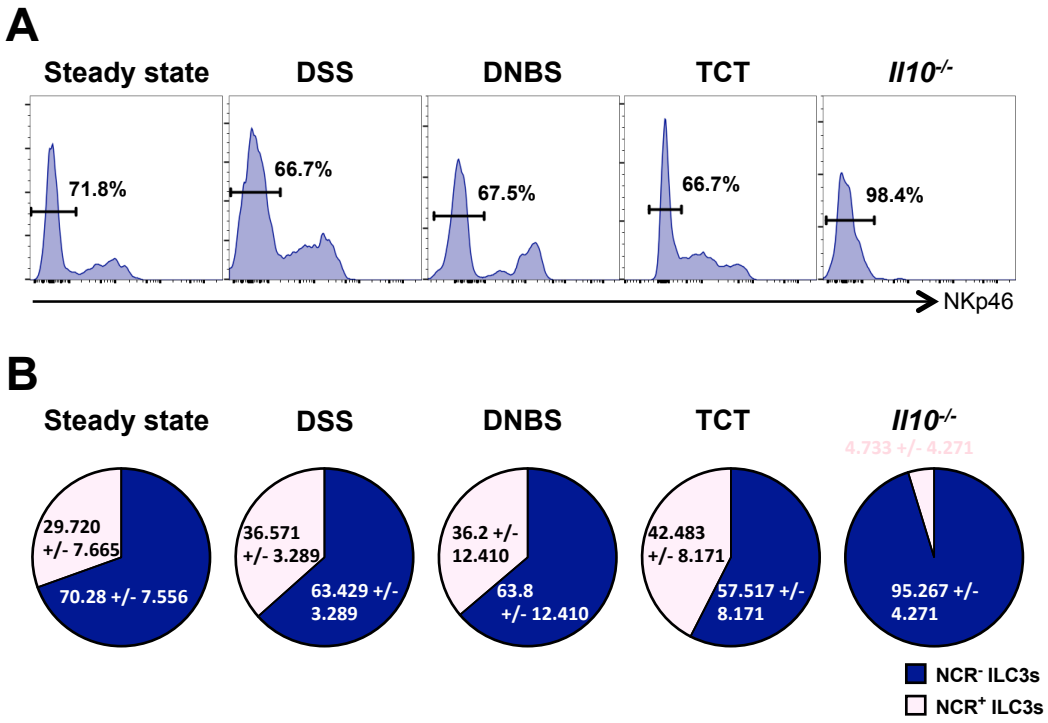

Figure 8: NCR$^-$ ILC3s are the dominant group 3 ILC subset in colonic LP in both health and disease. Experimental colitis was induced in genetically modified mice as shown in Figure 7, and mononuclear cells were isolated from the colonic LP of healthy (control) and diseased mice to identify by flow cytometry the percentages of NCR$^-$ ILC3s in health and disease, respectively. A. Histogram plots showing percentage of NCR$^-$ ILC3s in colonic LP in steady state and during intestinal inflammation using DSS, DNBS, TCT and *Il10*$^{-/-}$ model of experimental colitis, respectively. Cells were gated as single live CD45$^+$CD3$^-$IL-7R$^+$KLRG1$^-$RORγt$^+$NKp46$^-$ cells. Data representative of a single experiment with n=5 (Baseline), n=7 (DSS), n=8 (DNBS), n=6 (TCT) and n=6 (*Il10*$^{-/-}$). Data were analysed using FlowJo software (Treestar). B. Pie charts showing percentages (mean +/- SD) of NCR$^-$ and NCR$^+$ ILC3s in colonic LP in steady state, DSS, DNBS, TCT and *Il10*$^{-/-}$ model of experimental colitis, respectively. Data representative of a single experiment with n=5 (Baseline), n=7 (DSS), n=8 (DNBS), n=6 (TCT) and n=6 (*Il10*$^{-/-}$). Statistical analysis was performed using FlowJo software (Treestar) and GraphPad PRISM® version 7 software.

3.2 The majority of CD4 expressing group 3 ILCs are NCR$^-$ ILC3s

Since NCR$^-$ ILC3s may also express CD4, the frequency of CD4 expressing group 3 ILCs in the colonic LP was also investigated. Mononuclear cells were isolated from the LP of *Rorc*-eGFP reporter mice, stained with fluorochrome-conjugated antibodies against known ILC cell surface markers and analysed by flow cytometry. RORγt expressing ILC3s were identified using the gating strategy shown in Figure 6. In steady state, less than 30% of ILC3s express CD4 in the colon, the majority of which (~95%) are NCR$^-$ ILC3s (Figure 9). Similarly to steady state, CD4 expressing ILC3s accounted for less than 30% of the ILC3 population in all models of experimental colitis tested (DSS, DNBS, TCT and *Il10*$^{-/-}$) (data not shown).

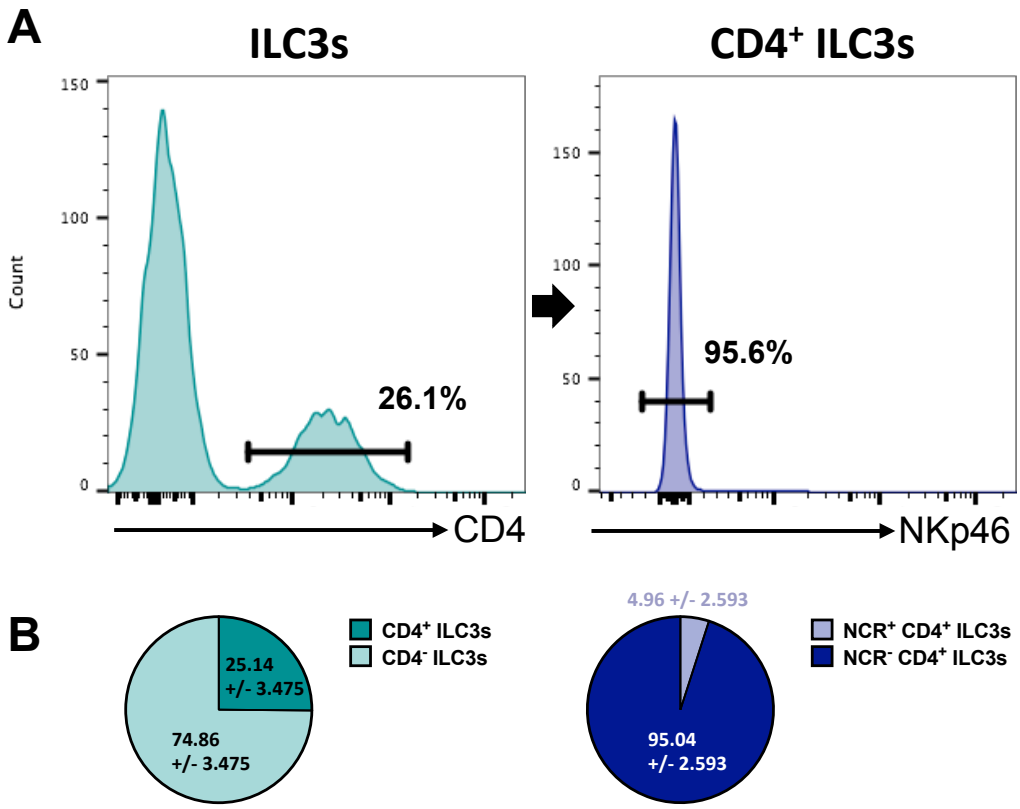

Figure 9: Less than 30% of colonic LP ILC3s express CD4 in steady state. Mononuclear cells were isolated from the colonic LP of healthy mice to identify by flow cytometry the percentage of $CD4^+$ ILC3s. A. Histogram plots showing percentages of $CD4^+$ ILC3s and NCR^-CD4^+ ILC3s in colonic LP in steady state. ILC3s were gated as single live $CD45^+CD3^-IL\text{-}7R^+KLRG1^-ROR\gamma t^+$ cells. Data representative of a single experiment with n=5. Data were analysed using FlowJo software (Treestar). B. Pie charts showing percentages (mean +/- SD) of $CD4^+$ ILC3s and NCR^-CD4^+ ILC3s in colonic LP in steady state respectively. Data representative of a single experiment with n=5. Analysis was performed using FlowJo software (Treestar) and GraphPad PRISM® version 7 software.

3.3 NCR$^-$ ILC3s are the dominant ILC3 subset in ILC mediated colitis

To selectively investigate the role of NCR$^-$ ILC3s in chronic colonic inflammation without the influence of other immune cells implicated in the pathology of IBD, *Tbx21*$^{-/-}$*Rag2*$^{-/-}$ Ulcerative Colitis (TRUC) mice were studied. This experimental model of chronic colitis resembles many aspects of human UC and offers a unique opportunity to selectively study NCR$^-$ ILC3s and their role in colitis. Due to *Rag2* deficiency B and T lymphocytes are absent, whereas ILC1s and NCR$^+$ ILC3s are impaired since their development depends on the transcription factor T-bet (Powell, Walker et al. 2012, Powell, Lo et al. 2015, Sciume, Hirahara et al. 2012, Klose, Flach et al. 2014). As shown in Figure 10, the percentage of ILC1s is less than one third of the percentage of ILC1s observed in TRUC mice (10.2% and 37.1%, respectively). Similarly, the percentage of NCR$^+$ ILC3s is also reduced in the colonic LP of TRUC mice compared to control *Rag2*$^{-/-}$ mice (Figure 10). However, without enumeration data, it is impossible to draw any conclusions regarding the absolute numbers of these cells in TRUC mice compared to control *Rag2*$^{-/-}$ mice. TRUC mice develop chronic colitis and previous studies have already established a central role for NCR$^-$ ILC3s in this disease (Powell, Walker et al. 2012, Powell, Lo et al. 2015, Ermann, Staton et al. 2014).

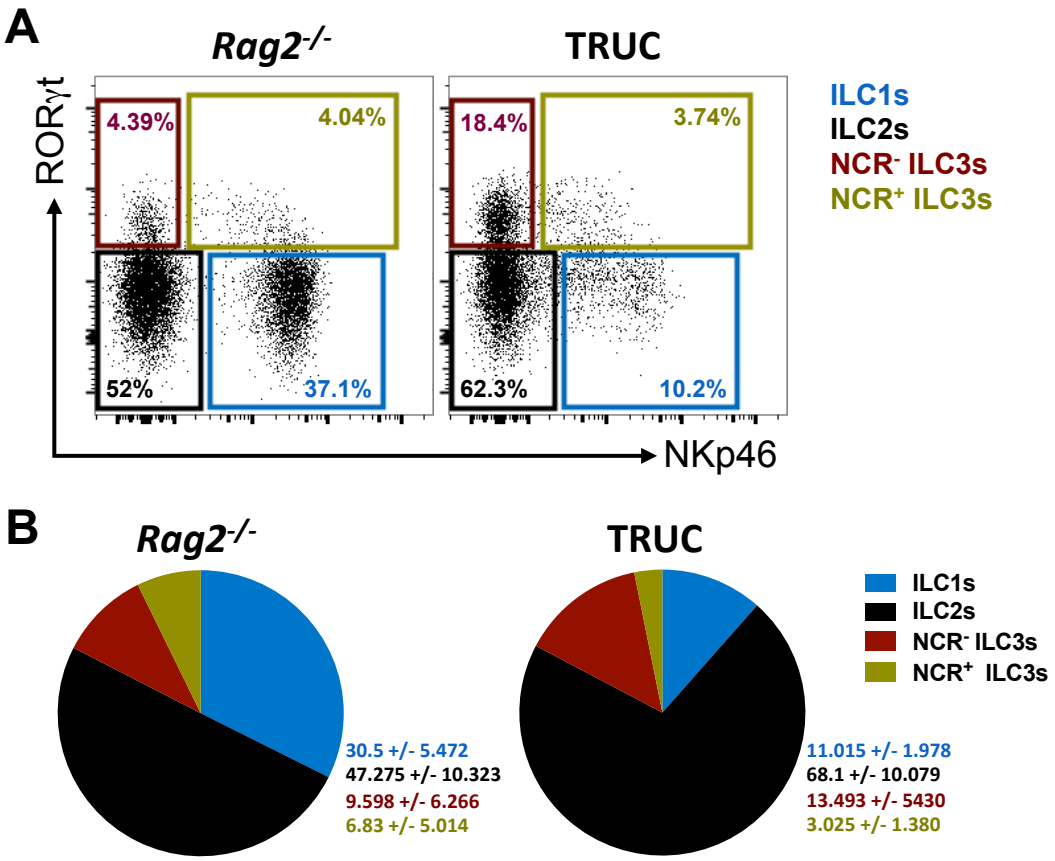

Figure 10: Reduced percentages of ILC1s and NCR$^+$ ILC3s in TRUC mice. cLPMCs were isolated from the colon of TRUC and *Rag2*$^{-/-}$ (control) mice to identify all ILC subpopulations by FACS. A. Flow dot plots showing the percentages of the different ILC subsets (as defined by their expression of RORγt and NKp46 in the colonic LP of *Rag2*$^{-/-}$ and TRUC 8-12 week old mice, respectively. Data representative of a single experiment with n=4. Analysing was performed using FlowJo software (Treestar). B. Pie charts depicting percentages (mean) of NCR$^-$ ILC3s, NCR$^+$ ILC3s, ILC1s and ILC2s in the colon of *Rag2*$^{-/-}$ and TRUC mice respectively. Data representative of a single experiment with n=4. Data were analysed using FlowJo software (Treestar) and GraphPad PRISM® version 7 software.

3.4 IL-22 responsive genes are highly expressed in TRUC disease

To further characterize NCR^{-} ILC3 mediated chronic colitis, transcriptional changes in the colon of TRUC mice were studied using microarray analysis. 1cm long fragments were obtained from the distal colon (~1cm away from the anal verge) of 8-12 week old *Rag2*$^{-/-}$ and TRUC mice. Tissue was homogenized, RNA was extracted, reverse transcribed, amplified and labelled for subsequent microarray analysis (MouseWG-6 v2.0 Expression BeadChip, Illumina). As expected, colonic tissue from TRUC mice showed a distinct transcriptional profile compared to the colonic tissue obtained from *Rag2*$^{-/-}$ mice as depicted by principal component analysis in Figure 11A. In total, seven hundred and eighty nine genes were differentially expressed (+/- 1.5 fold) in the colon of TRUC mice in comparison with the colon of *Rag2*$^{-/-}$ mice (Figure 11B). Gene set enrichment analysis (GSEA) on these genes performed by Dr Behdad Afzali revealed an enrichment of transcripts known to be regulated by IL-22 such as *Reg3b* (32.2 fold increase) and *Reg3g* (9.1 fold increase) in the colon of TRUC mice (Figure 11C).

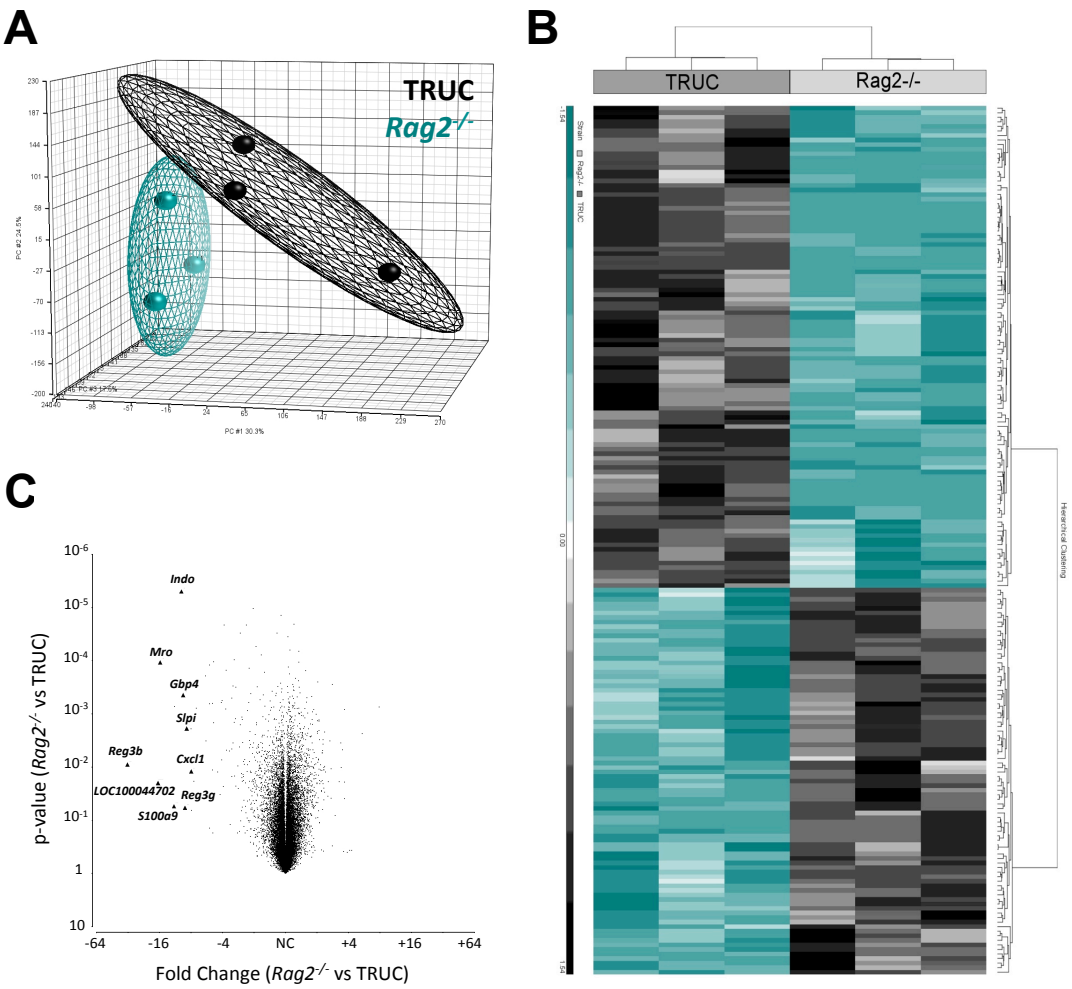

Figure 11: Transcriptional analysis of colonic tissue from TRUC vs. *Rag2*$^{-/-}$ (control) mice. 1cm long fragments of distal colon were harvested from 8-12 week old TRUC and *Rag2*$^{-/-}$ (control) mice and whole genome expression studies were performed using Illumina gene expression arrays. A. PCA of gene expression on distal colon of TRUC vs. *Rag2*$^{-/-}$ mice. Numbers along axes indicate relative scaling of the principal variables. Each sphere represents one mouse. Data representative of a single experiment with 3 biological replicates. Analysis was performed using Partek® software. B. Heat map and cluster dendogram showing deferentially (+/- 1.5 fold) expressed genes on TRUC vs. *Rag2*$^{-/-}$ (control) mice. Data representative of a single experiment with 3 biological replicates. Analysis was performed using Partek® software. C. Volcano plot (fold change plotted against statistical significance of change) depicting transcriptional changes in colon of TRUC vs. *Rag2*$^{-/-}$ (control) mice. Data representative of a single experiment with 3 biological replicates. Analysis was performed using Partek® software.

To interrogate the possibility of IL-22 driving an important transcriptional program in NCR$^-$ ILC3 mediated colitis, the expression pattern of IL-22 responsive genes was analysed in the colon of TRUC mice. IL-22R is known to be expressed in non-haematopoietic cell lineages such as epithelial and stromal cells (Wolk, Kunz et al. 2004). To characterize the expression profile of IL-22 receptor (IL-22R) in the colon, unfractionated cells were isolated from the colon of 8-12 week old WT mice, and stained with fluorochrome-conjugated Abs to allow epithelial/immune cell separation by flow cytometry. As shown in Figure 12, only epithelial cells (CD45$^-$EpCAM$^+$) but not immune cells (CD45$^+$EpCAM$^-$) express IL-22R in the murine colon. Therefore, an *in vitro* culture system of colonic epithelial organoids was established by Dr Anastasia Tsakmaki and Dr Gavin Bewick. Colonic crypt stem cells from WT mice were harvested and cultured to form three dimensional organ buds that retain the phenotypic and functional characteristics of intact primary colonic epithelial cells (data not shown).

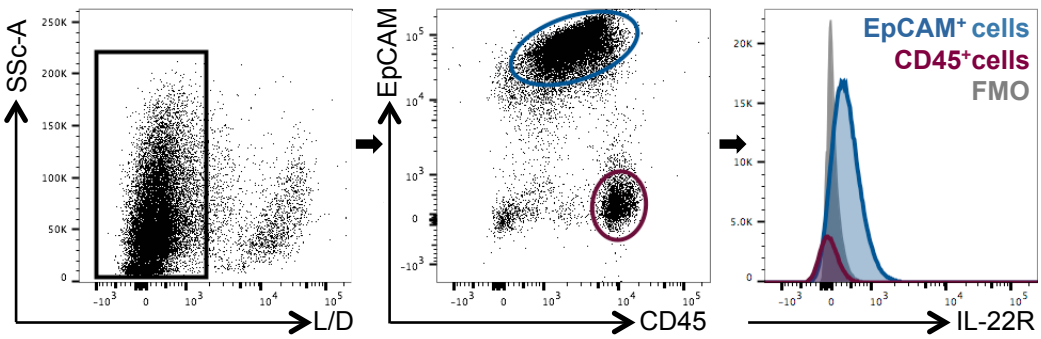

Figure 12: IL-22R expression is confined to colonic epithelial cells. Unfractionated cells were isolated from the colon of 8-12 week old WT mice and their expression of IL-22R was analysed by FACS. Flow dot plots (gated on single cells) showing gating strategy to define epithelial and immune cells in colonic LP of 8-12 week old WT mice and their IL-22R expression (histogram overlays). Data representative of 3 independent experiments with n=3-5.

Then, to define a profile of IL-22 responsive genes expressed in the colonic epithelium, colonoids were treated by Dr Anastasia Tsakmaki with or without 10ng/ml of murine recombinant IL-22 for a period of 24h. Transcriptional changes triggered by IL-22 treatment were identified by microarray analysis (Mouse Gene 2.0 ST array, Affymetrix). IL-22 treatment resulted in marked transcriptional changes in colonoids. In total, four hundred fifty one genes were significantly ($P<0.002$) differentially expressed (+/- 1.5 fold) in IL-22 treated colonoids compared to untreated ones (data not shown). Notably, amongst these genes were known IL-22 responsive genes such as *Reg3b* (187 fold increase, $P<1.96 \times 10^{-6}$), *Reg3g* (9.12 fold increase, $P<3 \times 10^{-6}$), *S100a8* ($P<9 \times 10^{-7}$), *S100a9* (11.6 fold increase, $P<2 \times 10^{-4}$), *Fut2* (2 fold increase, $P<6 \times 10^{-6}$) and *Saa3* (2.3 fold increase, $P<0.002$), as shown in Figure 13. Moreover, GSEA performed by Dr Behdad Afzali revealed that IL-22 responsive genes in colonic epithelial cells (as identified in IL-22 treated colonoids) were significantly enriched in the colon of TRUC mice ($P<0.0001$) (Figure 14A). Furthermore, as shown in Figure 14B, the expression of the IL-22 responsive genes found in colonoids significantly correlated with the expression level of these transcripts in the colon of TRUC mice ($r^2=0.66$, $P<0.0001$) (Analysis done by Dr Nicholas Powell). In particular, a comparison between the 30 most highly upregulated genes in TRUC mice and the top 30 upregulated genes in IL-22 treated colonoid revealed one third overlap (10 genes were shared), while a comparison between the 10 most highly upregulated genes in TRUC mice and the top 10 upregulated genes in IL-22 treated colonoid showed a 40% overlap (Figure 15) confirming the dominant IL-22 responsive gene signature in NCR^- ILC3 mediated colitis.

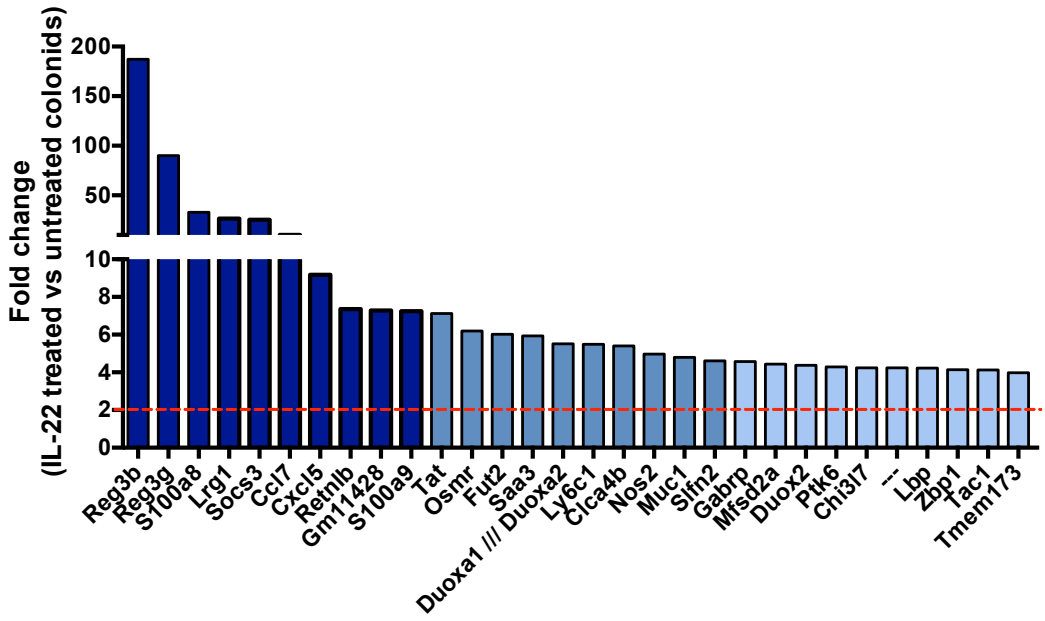

Figure 13: Top 30 of IL-22 responsive genes. Mouse colonoids (generated as described in Chapter 2) were stimulated with or without 10ng/ml IL-22 for 24h. To identify all differentially expressed genes whole transcriptomic analysis was performed using Mouse Gene 2.0 ST array, Affymetrix. IL-22 responsive genes were defined as genes upregulated in IL-22 treated colonoids when compared to untreated (control) colonoids. Graph showing fold change of the top 30 upregulated genes in IL-22 treated colonoids. Data representative of a single experiment with 3 technical replicates. Analysis was performed using Partek® and GraphPad PRISM® version 7 software.

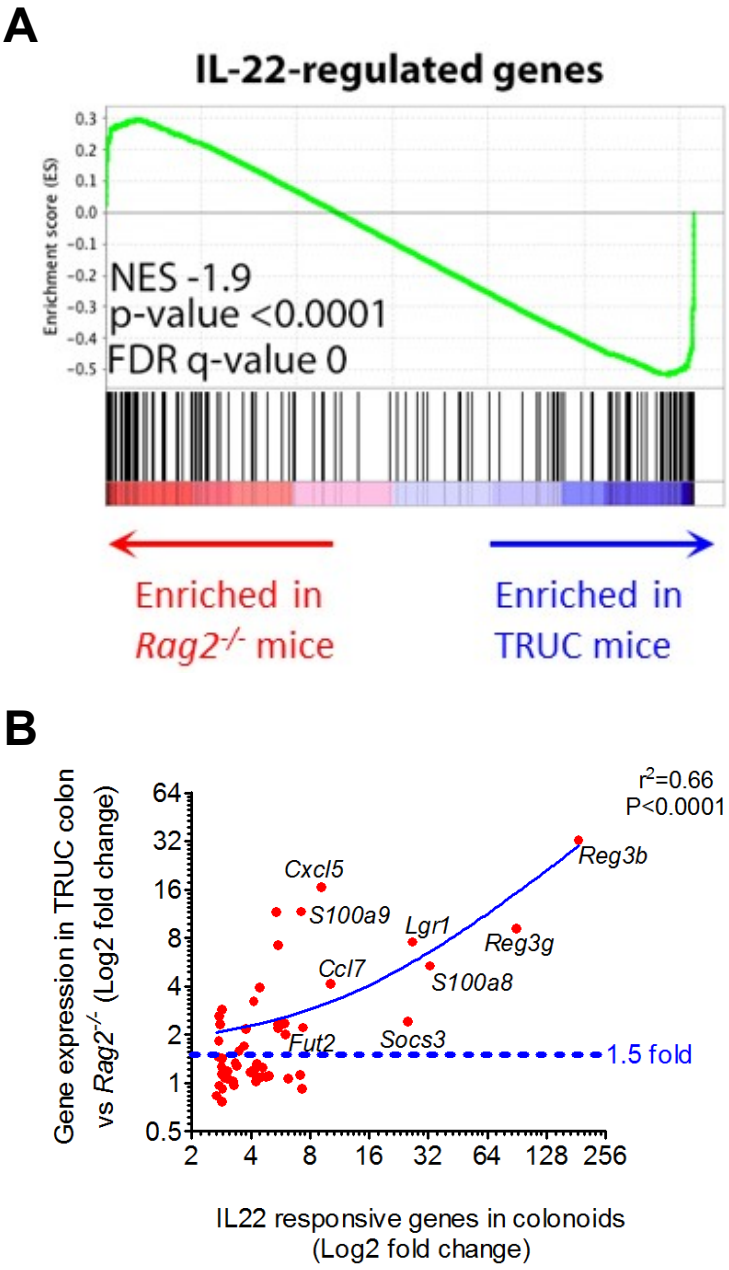

Figure 14: NCR⁻ ILC3 mediated colonic inflammation is characterised by a striking IL-22 responsive transcriptional profile. A. GSEA of IL-22 responsive genes in the colon of TRUC and *Rag2*$^{-/-}$ mice. IL-22 responsive genes were identified by microarray analysis as significantly ($p<0.05$) differentially expressed genes (+/-2 fold change) in colonic epithelial cells (colonoids) treated for 24h with or without 10ng/ml IL-22 (n=3) using the Mouse Gene 2.0 ST array (Affymetrix). This IL-22 responsive gene set was used to perform a GSEA in a whole genome expression array dataset of genes expressed in the distal colon of *Rag2*$^{-/-}$ (n=3) and TRUC (n=3) mice (MouseWG-6 v2.0 Expression BeadChip, Illumina). B. Correlation of the expression of the top 50 most highly upregulated IL-22 responsive genes in colonoids with their expression in the colon of TRUC mice. IL-22 responsive

genes were defined by whole genome microarray analysis of colonoids treated for 24h with or without 20ng/ml IL-22 (n=3). The expression of IL-22 responsive genes was then correlated with the expression of these genes in whole segments from distal colon of TRUC mice when compared to *Rag2*$^{-/-}$ (control) mice (n=3).

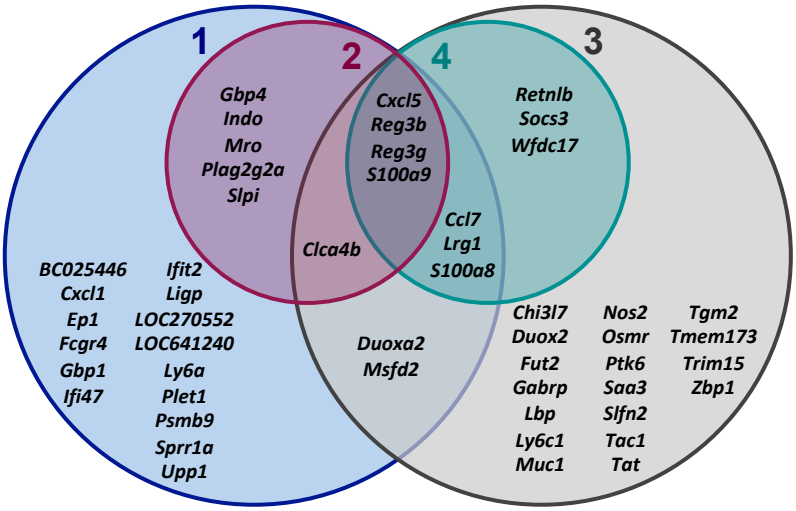

Figure 15: Shared expression of the top 30 (1, 3) and top 10 (2, 4) most highly upregulated genes in the colon of TRUC mice and IL-22 treated colonoids, respectively. 1cm long fragments of distal colon were harvested from 8-12 week old TRUC and *Rag2*$^{-/-}$ (control) mice and whole genome expression studies were performed using microarrays (MouseWG-6 v2.0 Expression BeadChip, Illumina). Highly upregulated genes in TRUC colon were defined as significantly ($p<0.05$) upregulated genes (+/- 1.5 fold) in TRUC vs. *Rag2*$^{-/-}$ (control colon) by microarray analysis. Colonic epithelial cells (colonoids) were treated for 24h with or without 10ng/ml IL-22 (n=3) and whole genome expression analysis was performed using Mouse Gene 2.0 ST array (Affymetrix). IL-22 responsive genes were identified as significantly ($p<0.05$) upregulated (+/- 1.5 fold change) in IL-22 treated vs. untreated (control) colonoids. Graph showing the top 30 (1, 3) and top 10 (2, 4) most highly upregulated genes in the colon of TRUC mice and IL-22 treated colonoids, respectively.

3.5 NCR$^-$ ILC3s are prominent producers of IL-22

In accordance with the dominant IL-22 responsive signature in NCR$^-$ ILC3 mediated colitis, previous work in the lab has shown that mononuclear cells isolated from the colonic LP (cLPMCs) of TRUC mice produced significantly more IL-22 and IL-17A compared to cLPMCs from *Rag2*$^{-/-}$ mice, and in absolute terms the IL-22 levels were substantially higher than the IL-17A ones. Taken together, these data point to a potential role for IL-22 in TRUC disease. Since neutrophils (Zindl, Lai et al. 2013) and other myeloid cells (Pickert, Neufert et al. 2009) also produce IL-22, the source of IL-22 in the colon of TRUC mice was investigated. Previous work in the lab using FACS analysis on cLPMCs isolated from TRUC mice showed that immune (CD45$^+$) cells that produce IL-22 in the colon of TRUC mice express CD90, a known ILC marker, whereas a substantial proportion of these cells also produce IL-17A, suggesting that ILCs are the main cellular source of IL-22 in TRUC disease.

To confirm that NCR$^-$ ILC3s and not ILC2s, the other major ILC subset in TRUC mice, were indeed the producers of IL-22 in TRUC disease, NCR$^-$ ILC3s and ILC2s were FACS sorted from the colonic LP of TRUC mice for subsequent whole transcriptome analysis by microarrays. As expected NCR$^-$ ILC3s and ILC2s from the colonic LP of TRUC mice showed distinct gene expression profiles (Figure 16A) that were similar to their corresponding small intestinal counterparts (Robinette, Fuchs et al. 2015). In total, four hundred and seventy genes were differentially expressed (+/- 1.5 fold, $P<0.05$) between NCR$^-$ ILC3s and ILC2s (Figure 16B), including ILC2 (*Gata3, Il5, il13*) and ILC3 (*il22*, *il17a*, *Il23r*) relevant genes, respectively (Figure 18C), with the *il22* gene being both the most highly upregulated and most significantly differentially expressed gene in NCR$^-$ ILC3s (13.6 fold increase, $P<0.005$). Moreover, stimulation with both IL-1β and IL-23 induced differential expression of 324 genes (+/- 1.5 fold, $P<0.05$) on FACS purified NCR$^-$ ILC3s, with *il22* being both the most highly upregulated and most significantly differentially expressed gene in activated NCR$^-$ ILC3s (Figure 17A). To corroborate these findings on protein level, FACS sorted NCR$^-$ ILC3s fro the colon of TRUC mice were stimulated with or without IL-1β and IL-23 for 24h, and IL-22 levels in the supernatant (S/N) were measured by ELISA. As shown in Figure 17B, although colonic NCR$^-$ ILC3s spontaneously produce IL-22, IL-1β and IL-23 stimulation

significantly increased IL-22 production by these cells. Taken together, these data show that IL-22 drives a dominant transcriptional program in NCR^- ILC3 mediated chronic colitis observed in TRUC mice.

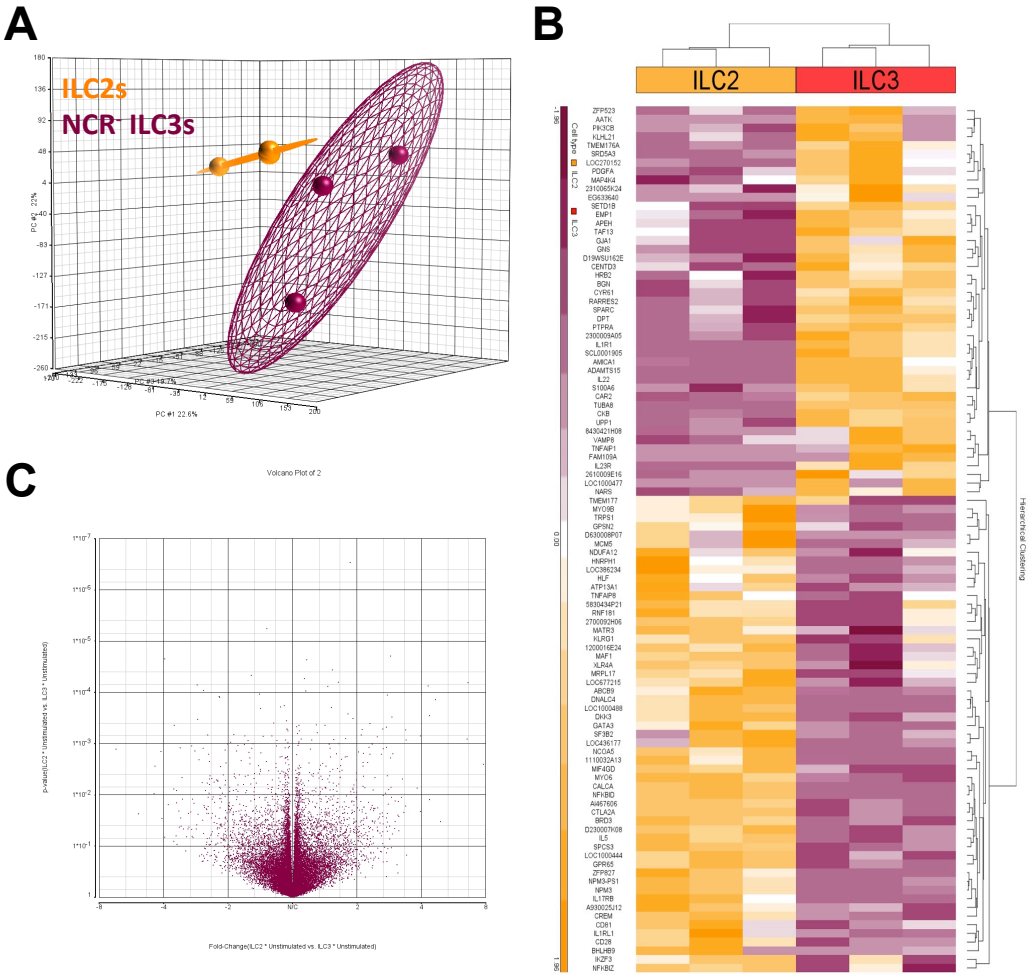

Figure 16: ILC2s and NCR$^-$ ILC3s show distinct gene expression profiles in the colon of TRUC mice. ILC2s and NCR$^-$ ILC3s were FACS purified from the colonic LP of 8-12 week old TRUC mice and whole genome expression studies were performed using Illumina gene expression arrays. A. PCA of gene expression on distal colon of ILC2s vs. NCR$^-$ ILC3s. Numbers along axes indicate relative scaling of the principal variables. Each sphere represents one technical replicate. Data representative of a single experiment with 3 technical replicates. Analysis was performed using Partek® software. B. Heat map and cluster dendogram showing deferentially (+/- 2 fold) expressed genes on ILC2s vs. NCR$^-$ ILC3s purified from the colonic LP of TRUC mice. Data representative of a single experiment with 3 technical replicates. Analysis was performed using Partek® software. C. Volcano plot (fold change plotted against statistical significance of change) depicting transcriptional changes between ILC2s and NCR$^-$ ILC3s purified from the colonic LP of TRUC mice. Data representative of a single experiment with 3 technical replicates. Analysis was performed using Partek® software.

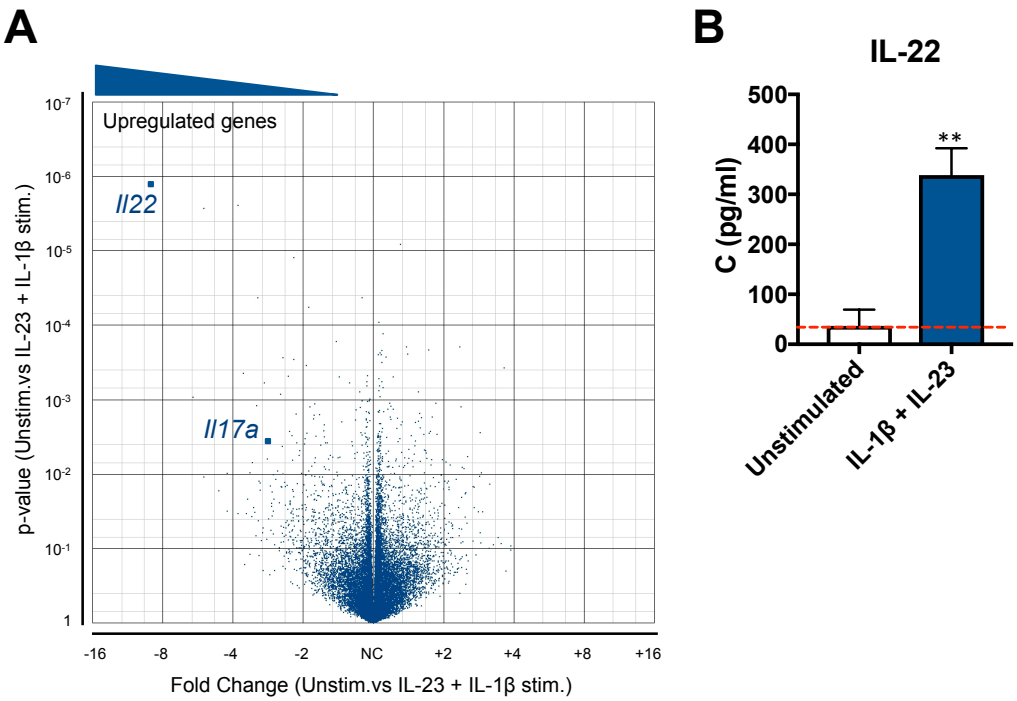

Figure 17: NCR⁻ ILC3s are prominent producers of IL-22 in the TRUC model of experimental colitis. NCR⁻ ILC3s were FACS sorted from the colon of TRUC mice and stimulated for 24h with or without 20ng/ml IL-23 or 10ng/ml IL-1β. Cells were the lysed, RNA was extracted and transcriptome analysis was performed using Illumina gene expression arrays. A. Volcano plot (fold change plotted against statistical significance of change) showing transcriptional differences on FACS purified NCR⁻ ILC3s stimulated (24h) with or without IL-23 and IL-1β (n=3 in each group). Data representative of a single experiment with 3 technical replicates. Analysis was performed using Partek® software. B. IL-22 production (mean with SD) in S/Ns of FACS sorted NCR⁻ ILC3s from the colon of 8-12 week old TRUC mice. Cells were stimulated for 24h with (n=3) or without IL-23 and IL-1β (n=2) and IL-22 production was measured by ELISA. Data polled from 2 independent experiments. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney two-tailed, p=0.0095).

3.6 IL-22 drives inflammation in TRUC mice

The striking IL-22 transcriptional signature observed in TRUC disease strongly suggests a functional role for this cytokine in NCR$^-$ ILC3 mediated chronic colitis. To interrogate this possibility, and taking into account recent data highlighting the importance of IL-22 in restoring intestinal epithelial homeostasis (Lindemans, Calafiore et al. 2015), *Tbx21*$^{-/-}$*Rag2*$^{-/-}$*Il22*$^{-/-}$ (TRUC*Il22*$^{-/-}$) triple knock out (KO) mice were generated. Surprisingly, in contrast to TRUC mice that develop disease characterized by colitis, increased colonic weight and splenomegaly, TRUC*Il22*$^{-/-}$ mice did not develop spontaneous colitis (data not shown). As shown by Powell et al. in 2012, the intestinal microbiota composition plays a central role in TRUC disease, so TRUC*Il22*$^{-/-}$ mice were orally gavaged with intestinal microbiota harvested from TRUC mice to access whether exposure to "colitogenic" microbiota would impact disease susceptibility. Even then, TRUC*Il22*$^{-/-}$ mice remained disease free. TRUC*Il22*$^{-/-}$ mice exhibited 0 histological score as defined by H&E staining (Figure 18A and 18B) and had significantly smaller spleens and colons, compared to TRUC mice (Figure 18C). In addition, TRUC*Il22*$^{-/-}$ mice had significantly less immune infiltration in secondary lymphoid tissues such as spleen and MLN, as well as in the colonic LP (Figure 18D).

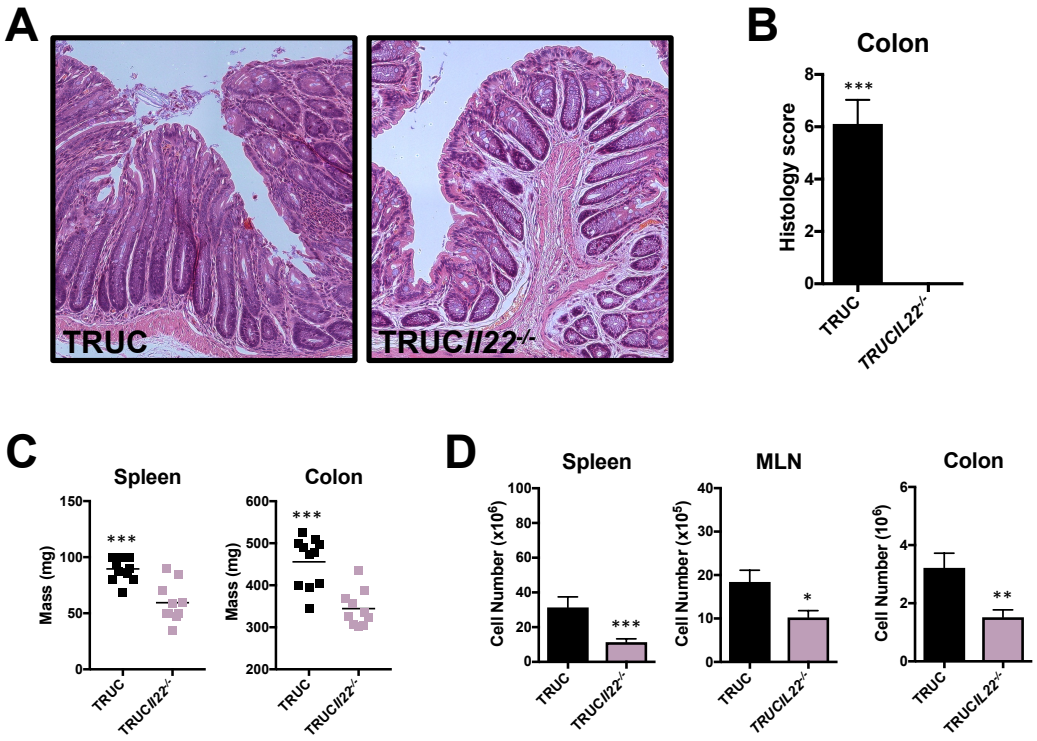

Figure 18: IL-22 is pathogenic in TRUC disease. TRUC and TRUC*Il22*$^{-/-}$ mice were orally gavaged with intestinal microbiota at day 0 and culled 6 weeks later at day 42. Spleen and colon masses were recorded, tissue fragments were obtained from the distal colon for histological analysis, and single cells suspensions were prepared from spleens, MLNs and colons to measure immune infiltrate. A. H&E staining on sections from the distal colon of TRUC and TRUC*Il22*$^{-/-}$ mice. B. Histology score (mean with SD) of TRUC and TRUC*Il22*$^{-/-}$ mice. Data pooled from two independent experiments with n=5 and n=6 respectively. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney two-tailed test). C. Spleen and colon weights (line depicts mean) of TRUC vs. TRUC*Il22*$^{-/-}$ mice. Each square represents one mouse. Data pooled from two independent experiments with n=5 and n=6 respectively. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney two-tailed test). D. Total cell counts in spleen, MLN and colon (mean with SD) of TRUC and TRUC*Il22*$^{-/-}$ mice, respectively. Data pooled from two independent experiments with n=5 and n=6 respectively. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

To address whether blocking of IL-22 *in vivo* could ameliorate disease in TRUC mice, aIL-22 neutralizing Abs were administered intraperitoneally (*ip.*) to TRUC mice every three to four days, and mice were culled at day 27 (Figure 19A) for subsequent analysis. As shown in Figure 19B and 19C, *in vivo* blockade of IL-22 attenuated TRUC disease, with treated mice exhibiting zero histological score. Moreover, aIL-22 treated mice had significantly smaller organ masses compare to TRUC mice treated with isotype control antibodies (Figure 19D).

In accordance with IL-22 having a key role in NCR^{-} ILC3 mediated chronic colitis, *in vivo* administration of recombinant IL-22 to TRUC*Il22*$^{-/-}$ mice induced disease to otherwise protected TRUC*Il22*$^{-/-}$ mice (Figure 20). TRUC*Il22*$^{-/-}$ mice treated for a period of 14 days as shown in Figure 20A, had significantly higher histological score than TRUC*Il22*$^{-/-}$ mice treated with PBS (Figure 20B and 20C). Furthermore, TRUC*Il22*$^{-/-}$ mice had significantly larger colon masses compared to PBS treated mice, although no difference in the spleen sizes was observed (Figure 20D). Taken together, these data point to IL-22 having an important role in the pathogenesis of TRUC disease.

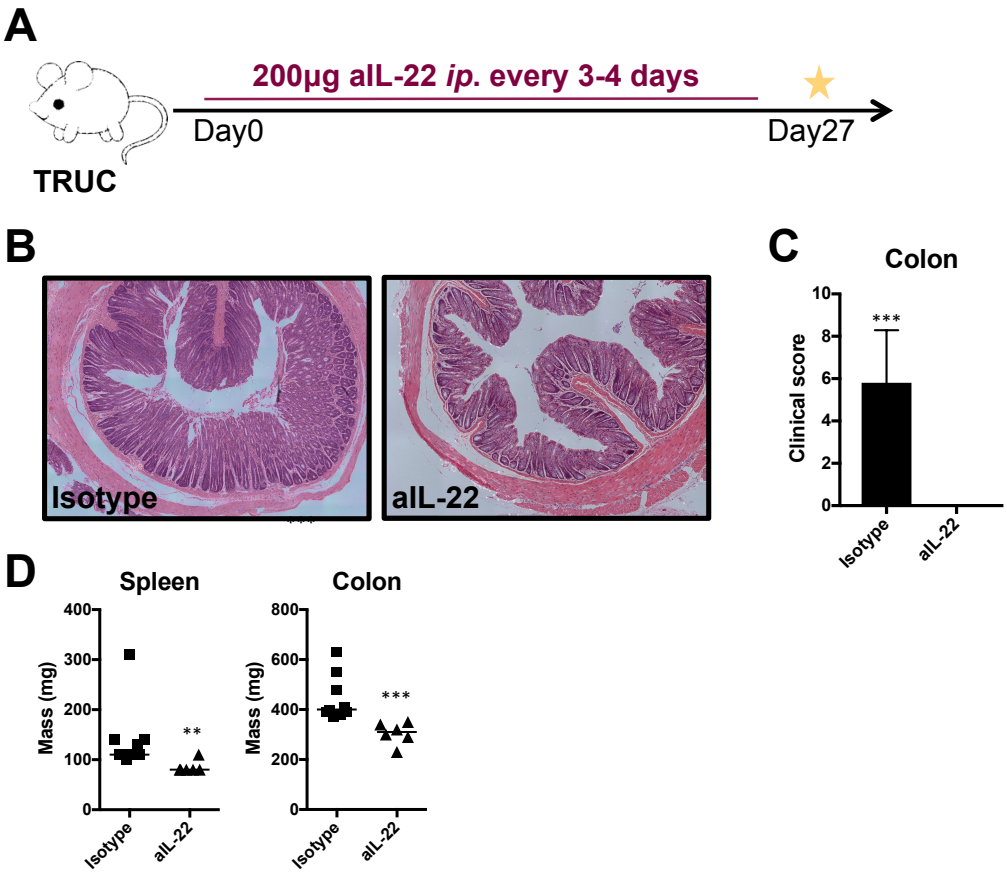

Figure 19: IL-22 blockade attenuates TRUC disease. TRUC mice were administered *ip.* with 200µg aIL-22 or isotype antibody (control) and seven days later were culled for downstream analysis. Spleen and colon masses were recorded and tissue fragments were obtained from the distal colon for histological analysis. A. Experimental protocol for *in vivo* administration of aIL-22 or isotype control Ab in TRUC mice. B. H&E staining of distal colon of TRUC mice treated with isotype control and aIL-22 respectively. Data representative of a single experiment. C. Histology score (mean with SD) of TRUC mice treated with isotype control and aIL-22 respectively. Data representative of a single experiment. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney two-tailed test). D. Spleen and colon weights (line depicts mean) of isotype treated vs. aIL-22 treated TRUC mice. Each square represents one mouse. Data representative of a single experiment. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney one-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

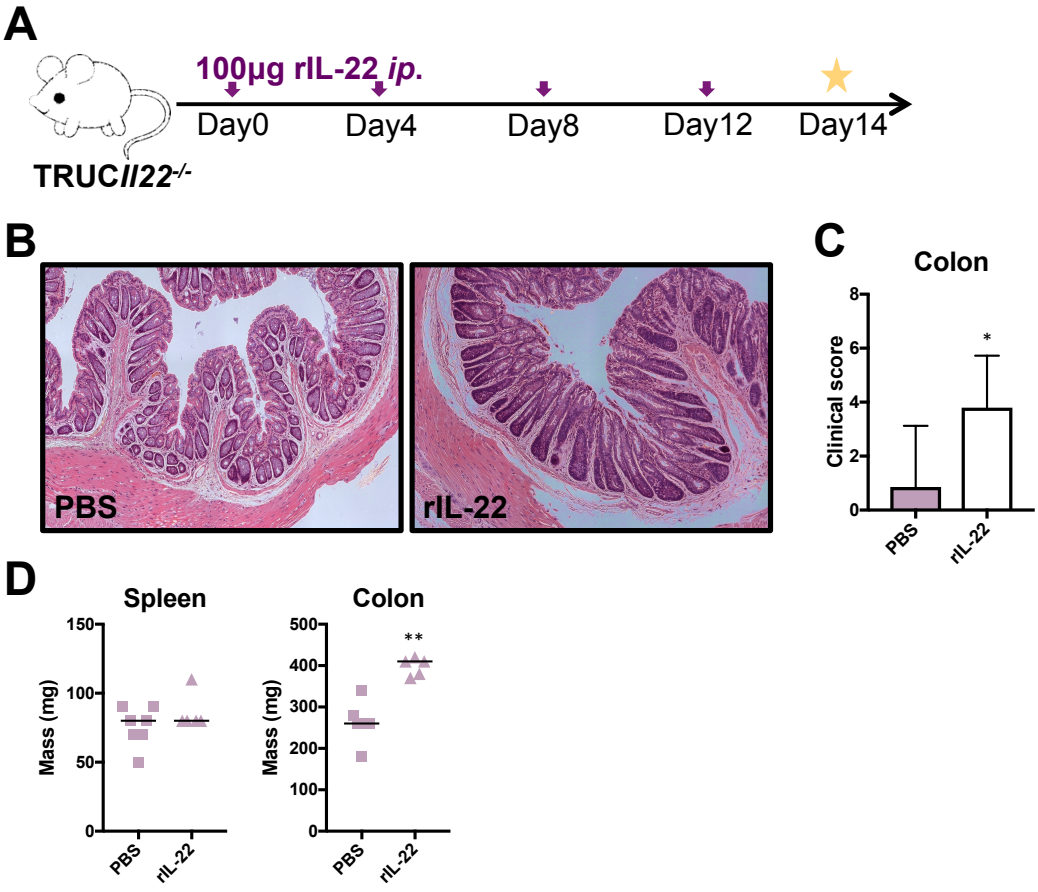

Figure 20: rIL-22 induces disease in TRUC*Il22*$^{-/-}$ mice. TRUC*Il22*$^{-/-}$ mice were administered *ip.* with 100µg rIL-22 or PBS (control) at days 0, 4, 8 and 12, and were culled at day 14 for downstream analysis. Spleen and colon masses were recorded and tissue fragments were obtained from the distal colon for histological analysis. A. Experimental protocol for *in vivo* administration of rIL-22 or PBS (control) in TRUC*Il22*$^{-/-}$ mice. B. H&E staining on sections from the distal colon of TRUC*Il22*$^{-/-}$ mice treated with PBS and rIL-22 respectively. Data representative of a single experiment. C. Histology score (mean with SD) of TRUC*Il22*$^{-/-}$ mice treated with PBS and rIL-22 respectively. Data representative of a single experiment. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney two-tailed test). D. Spleen and colon weights (line depicts mean) of PBS vs. rIL-22 treated TRUC*Il22*$^{-/-}$ mice. Each square represents one mouse. Data representative of a single experiment. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney one-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

3.7 Discussion

In the first results chapter, it is shown that NCR$^-$ ILC3s is the most abundant ILC3 subset in the colonic LP in steady state, as well as during intestinal inflammation. Several independent studies have described an important role for ILC3s in host immunity to foreign pathogens and commensal bacteria in the gut (Sonnenberg, Monticelli et al. 2012, Hepworth, Monticelli et al. 2013), but only a minority of those were focused on NCR$^-$ ILC3s (Buonocore, Ahern et al. 2010, Powell, Walker et al. 2012). Here, it is shown that NCR$^-$ ILC3s are the majority of ILC3s in the murine colon, and their large proportion amongst ILC3s is maintained upon induction of intestinal inflammation using several mouse models of experimental IBD, such as DSS and DNBS induced colitis, T cell transfer induced colitis, and *Il10*$^{-/-}$ mice. Moreover, further analysis showed that only a small percentage of ILC3s (around 30%) expresses the surface marker CD4, and that the vast majority (>95%) of those cells are NCR$^-$ ILC3s. However, it's worth mentioning here that although these experiments showed clearly that NCR$^-$ ILC3s are the major ILC3 subpopulation in the murine colon both in health and disease, they were only performed once with $4<n<8$, and thus, they need to be repeated in order to confirm these preliminary findings. Moreover, as there are no enumeration data in these experiments it is difficult to draw any meaningful conclusions about the absolute numbers of NCR$^-$ ILC3s in the colonic LP and how (if at all) are affected during intestinal inflammation.

As described previously, the TRUC mouse is an ideal model to study NCR$^-$ ILC3s due to the lack of adaptive immunity caused by *Rag2* deficiency, as well as the impaired percentages of ILC1s and NCR$^+$ ILC3s, the development of which depends on the transcription factor T-bet (Powell, Walker et al. 2012, Powell, Lo et al. 2015, Sciume, Hirahara et al. 2012, Klose, Flach et al. 2014). To be expected, NCR$^-$ ILC3s were also found to be the most abundant ILC3 subset in the colon of TRUC mice. In this chapter, it was shown that the percentages of both ILC1s and NCR$^+$ ILC3s were reduced in the colonic LP of TRUC mice compared to *Rag2*$^{-/-}$ control mice. Again, since the role of T-bet in the development of ILC1s and NCR$^+$ ILC3s is well characterized (Powell, Walker et al. 2012, Powell, Lo et al. 2015, Sciume, Hirahara et al. 2012, Klose, Flach et al. 2014), this experiment was only performed once with

n=4 and without obtaining any enumeration data. Thus, repeating this experiment, including absolute numbers of all ILC subsets found in the colonic LP of TRUC mice, would be extremely informative for the work described in this thesis.

Previous work in the lab and other independent groups have already established a role for ILC3s in TRUC disease (Powell, Walker et al. 2012, Powell, Lo et al. 2015, Ermann, Staton et al. 2014), therefore TRUC mice were exploited to scrutinize the role of NCR^- ILC3s in chronic colonic inflammation, which is the aim of this thesis. Transcriptome analysis in whole tissue from the colon of TRUC and *Rag2*$^{-/-}$ control mice revealed a distinct IL-22 transcriptional signature in TRUC disease. Transcripts known to be regulated by IL-22 such as those encoding for antimicrobial peptides (*Reg3b*, *Reg3g*) were increased in the colon of TRUC mice, corroborating the well-characterized role of IL-22 in host's defence against bacterial pathogens (Sonnenberg, Monticelli et al. 2012, Hepworth, Monticelli et al. 2013). Since IL-22 acts solely on the intestinal epithelium and not on other immune cells in the gut (Wolk, Kunz et al. 2004), mouse colonoids were developed by our collaborator Dr Anastasia Tsakmaki in order to define the transcriptional changes enforced by IL-22 in colonic epithelial cells. IL-22 stimulation induced notable transcriptional changes in murine colonoids, and as revealed by GSEA kindly performed by Dr Behdad Afzali these transcripts were enriched in the TRUC colon. Interestingly, the expression of these transcripts in IL-22 treated colonoids was positively correlated with their expression in the TRUC colon as shown by Dr Nick's Powell analysis. Taken together these findings indicate for the first time that IL-22 may have a role in TRUC disease.

Indeed, cLPMCs isolated form the colon of TRUC mice produced significantly more IL-22 than those isolated from the colon of *Rag2*$^{-/-}$ control mice. Moreover, previous work in the lab by FACS analysis on cLPMCs from TRUC mice revealed that the vast majority of IL-22 found in the colon of these mice came from cells expressing CD90, a known ILC marker, suggesting that ILCs are the main producers of IL-22 in the colonic LP in TRUC disease. Transcriptome analysis on FACS purified unstimulated ILC2s and NCR^- ILC3s from the colon of TRUC mice, showed *Il22* as the most highly upregulated, as well as most significantly expressed gene found on NCR^- ILC3s. IL-23 and IL-1β are known inducers of IL-22 production (Kastelein,

Hunter et al. 2007, Lee, Kumagai et al. 2013), transcriptome analysis on FACS sorted unstimulated and IL-23 and IL-1β stimulated NCR$^-$ ILC3s from the colonic LP of TRUC mice showed significantly increased expression of *Il22* in treated NCR$^-$ ILC3s. These findings were also corroborated in protein level, as it was shown that FACS sorted NCR$^-$ ILC3s from TRUC mice produced significantly more IL-22 upon 24h stimulation with IL-23 and IL-1β. These findings support further the hypothesis that IL-22 may be an important effector cytokine in TRUC disease.

Indeed, in this chapter it was shown for the first time that IL-22 drives inflammation in TRUC disease. Genetic depletion of IL-22 in TRUC mice protected them from colitis. In accordance with these findings, in vivo blockade of IL-22 by administration of neutralizing antibodies to TRUC mice significantly attenuated colonic inflammation, whereas in vivo administration of recombinant IL-22 to TRUC*Il22*$^{-/-}$ mice induced colitis in otherwise protected TRUC*Il22*$^{-/-}$ mice. Taken together these data challenge our current understanding of the actions of IL-22 in the gut, where is mainly viewed as a promoter of tissue regeneration and repair (Lindemans, Calafiore et al. 2015). Several independent studies have shown a protective role for IL-22 during acute epithelial injury caused by chemical insults such as DSS (Sugimoto, Ogawa et al. 2008) or methotrexate, a known chemotherapy agent (Aparicio-Domingo, Romera-Hernandez et al. 2015). In addition, IL-22 is known to be protective against self-limiting colonic infections as those caused by *Citrobacter rodentium* (Sanos, Bui et al. 2009, Satoh-Takayama, Vosshenrich et al. 2008). The results described in this chapter, somehow contradict our current views on IL-22 and its beneficial actions in the intestinal mucosa, pointing for the first time to a pro-inflammatory role for this cytokine. However, these findings were observed using TRUC mice, a model of chronic colitis where by definition tissue injury is on-going and inflammation doesn't reach a resolution phase, suggestion that the action of IL-22 might be context dependent. Furthermore, the absence of adaptive immunity in this system, may have also contributed to the pro-inflammatory actions of IL-22 described in this chapter. Thus, whether IL-22 is beneficial or detrimental during chronic inflammation in an immune competent organism is yet to be clear.

Finally, Powell *et al* have already established a key role for ILC3s in TRUC disease, which is at least partially mediated by IL-17A production (Powell, Walker et al.

2012). The results described in chapter 3 of this thesis implicate for the first time IL-22 (another cytokine that is co-produced along with IL-17A by NCR^- ILC3s) in TRUC pathogenesis suggesting for the first time that IL-22 may have a pro-inflammatory role during chronic colitis and that NCR^- ILC3s might use more than one mechanisms to drive pathology in TRUC disease.

Chapter 4

Results: IL-22 may drive inflammation in TRUC disease through ER stress induction in colonic epithelial cells

In the previous chapter it was shown that NCR$^-$ ILC3s drive colitis in TRUC mice through the production of IL-22 providing new insights into TRUC pathogenesis, although how exactly IL-22 regulate disease in these mice remains unclear. Therefore, this chapter aims to shed some light into how IL-22 regulates intestinal inflammation in the TRUC model of chronic colitis.

Emerging data now link endoplasmic reticulum (ER) stress with IBD pathogenesis (Kaser, Lee et al. 2008, Bertolotti, Wang et al. 2001, Zhang, Chen et al. 2015, Li, Zhang et al. 2017, Das, Png et al. 2013). Intestinal epithelial cells such as Paneth cells and Goblet cells are highly secretory cells, and thus prone to ER stress (Kaser, Lee et al. 2008, McGuckin, Eri et al. 2010, Todd, Lee et al. 2008). ER stress generally occurs when either unfolded or misfolded proteins accumulate in the ER (Kaser, Martinez-Naves et al. 2010, Kaser, Adolph et al. 2013). To avoid or minimize ER stress, an important cellular process is generated known as unfolded protein response (UPR) (Todd, Lee et al. 2008), which is particularly crucial to cells in the gastrointestinal tract (Kaser, Lee et al. 2008, McGuckin, Eri et al. 2010). As several independent studies have shown associations between IBD susceptibility and components of the UPR (Kaser, Lee et al. 2008, Shkoda, Ruiz et al. 2007, Deuring, de Haar et al. 2012), the hypothesis that IL-22 may drive colitis in TRUC mice through ER stress induction in colonic epithelial cells was tested in this chapter.

4.1 IL-22 induced the expression of known ER stress genes in colonic epithelial cells

Initially, to identify any specific pathways that IL-22 may imprint on the colonic epithelium, the pool of genes that were upregulated in IL-22 treated colonoids

compared to untreated ones were further interrogated. Strikingly, IL-22 treatment significantly increased the number of transcripts encoding for ER stress genes in mouse colonoids (Figure 21), which is in accordance with studies implicating ER stress in IBD pathogenesis (Kaser, Lee et al. 2008, Bertolotti, Wang et al. 2001, Zhang, Chen et al. 2015, Li, Zhang et al. 2017, Das, Png et al. 2013).

Therefore, in order to test the hypothesis of IL-22 inducing ER stress in colonic epithelial cells, mouse colonoids were treated with Tunicamycin-a known ER stress inducer (Das, Png et al. 2013)- by our collaborator Dr Anastasia Tsakmaki and as expected, Tunicamycin significantly induced the transcription of several ER stress genes including *Grp78* and *sXbp1* ($p<0.0001$ and $p<0.001$ respectively) in mouse colonoids (Figure 22), which were also induced following stimulation with IL-22 (Figure 21), suggesting that IL-22 may induce ER stress in colonic epithelial cells. However, its worth mentioning here that induction of some known ER stress by IL-22 in colonic epithelial cells is not necessarily indicative of ER stress presence in these cells, and thus further experiments are needed to interrogate the possibility of IL-22 mediated ER stress induction in colonic epithelial cells.

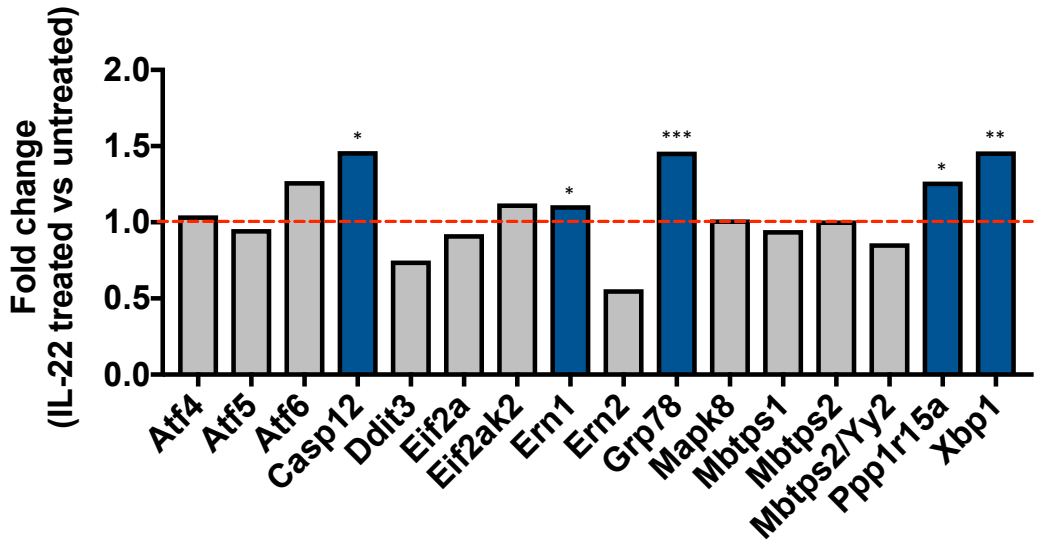

Figure 21: ER stress gene expression in IL-22 treated colonoids. Mouse colonoids were stimulated for 24h with or without 10ng/ml IL-22 for transcriptome analysis by microarrays (Mouse Gene 2.0 ST array, Affymetrix). Graph showing fold change in gene expression of known ER stress genes defined by microarray analysis. Blue bars represent significantly differentially expressed transcripts. Data representative of a single experiment with 3 technical replicates. Statistical analysis was performed using Partek® and GraphPad PRISM® version 7 software (ANOVA, *p<0.05, **P<0.005, ***p<0.0001).

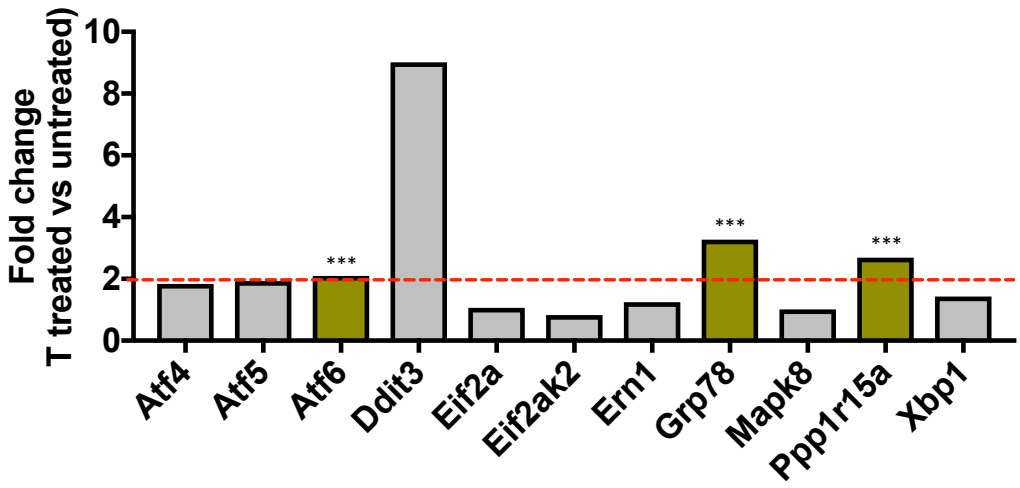

Figure 22: ER stress gene expression in Tunicamycin treated colonoids. Mouse colonoids were stimulated for 24h with or without Tunicamycin for transcriptome analysis by microarrays (Mouse Gene 2.0 ST array, Affymetrix). Graph showing fold change in gene expression of known ER stress genes defined by microarray analysis. Highlighted bars represent significantly upregulated genes. Data representative of a single experiment with 3 technical replicates. Statistical analysis was performed using Partek® and GraphPad PRISM® version 7 software (ANOVA, *p<0.05, **P<0.005, ***p<0.0001).

4.2 ER stress genes were significantly upregulated in the colon of TRUC mice

Subsequently, to formally investigate whether IL-22 drives colitis in TRUC mice through IL-22 mediated induction of ER stress in the colonic epithelium, gene expression microarray data on whole colonic tissue from TRUC and *Rag2*$^{-/-}$ control mice were further interrogated focusing on ER stress pathways. Interestingly, transcripts encoding known ER stress genes such as *Grp78*, *sXbp1* and *Atf6* were significantly upregulated in TRUC colon (Figure 23), supporting the hypothesis that IL-22 might drive TRUC disease through induction of ER stress in colonic epithelial cells. However, as mentioned before, additional experiments are required to confirm ER stress in the colonic epithelium of TRUC mice and subsequently its link to IL-22.

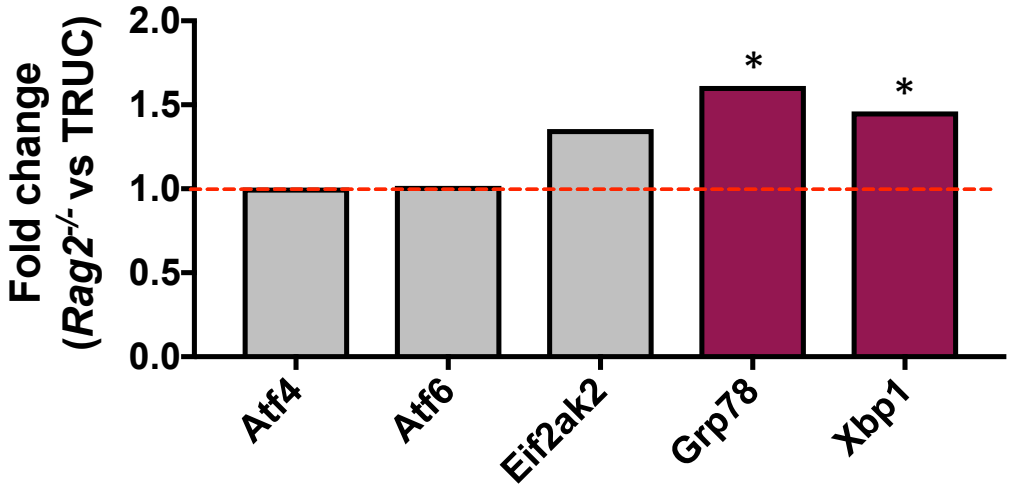

Figure 23: ER stress gene expression in the colon of TRUC mice. 1cm long fragments of distal colon were harvested from 8-12 week old TRUC and *Rag2*$^{-/-}$ (control) mice and whole transcriptome analysis was performed using microarrays (MouseWG-6 v2.0 Expression BeadChip, Illumina). Graph showing fold change of known ER stress genes upregulated in the colon of TRUC vs. *Rag2*$^{-/-}$ mice as defined by microarray analysis. Highlighted bars represent significantly upregulated genes. Data representative of a single experiment with 3 biological replicates. Statistical analysis was performed using Partek® and GraphPad PRISM® version 7 software (ANOVA, *p<0.05, **P<0.005, ***p<0.0001).

4.3 IL-22 produced by NCR$^-$ ILC3s induced expression of ER stress genes in colonic epithelial cells

Accordingly, to formally test the hypothesis of NCR$^-$ ILC3s driving TRUC disease via IL-22 mediated induction of ER stress, NCR$^-$ ILC3s were FACS sorted as described in chapter 2 from the colon of TRUC and TRUC*Il22*$^{-/-}$ mice respectively, activated through 48h stimulation with IL-2, IL-7, plus IL-23 and IL-1β, two known positive regulators of IL-22 (Kastelein, Hunter et al. 2007, Lee, Kumagai et al. 2013), followed by a 24h co-culture with colonoids as shown in Figure 24A. At the end of the 24h stimulation, mRNA levels of *Grp78*, *sXbp1* and *Grp94* were measured by RT-qPCR by Dr Anastasia Tsakmaki. As depicted in Figure 24B, *Grp78*, *sXbp1* and *Grp94* expression were significantly reduced in colonoids that were co-cultured with IL-22 deficient NCR$^-$ ILC3s compared to the levels in colonoids co-cultured with IL-22 sufficient NCR$^-$ ILC3s. Taken together, this data further support the hypothesis that NCR$^-$ ILC3s may drive inflammation in TRUC mice through IL-22 mediated induction of ER stress in the colonic epithelium.

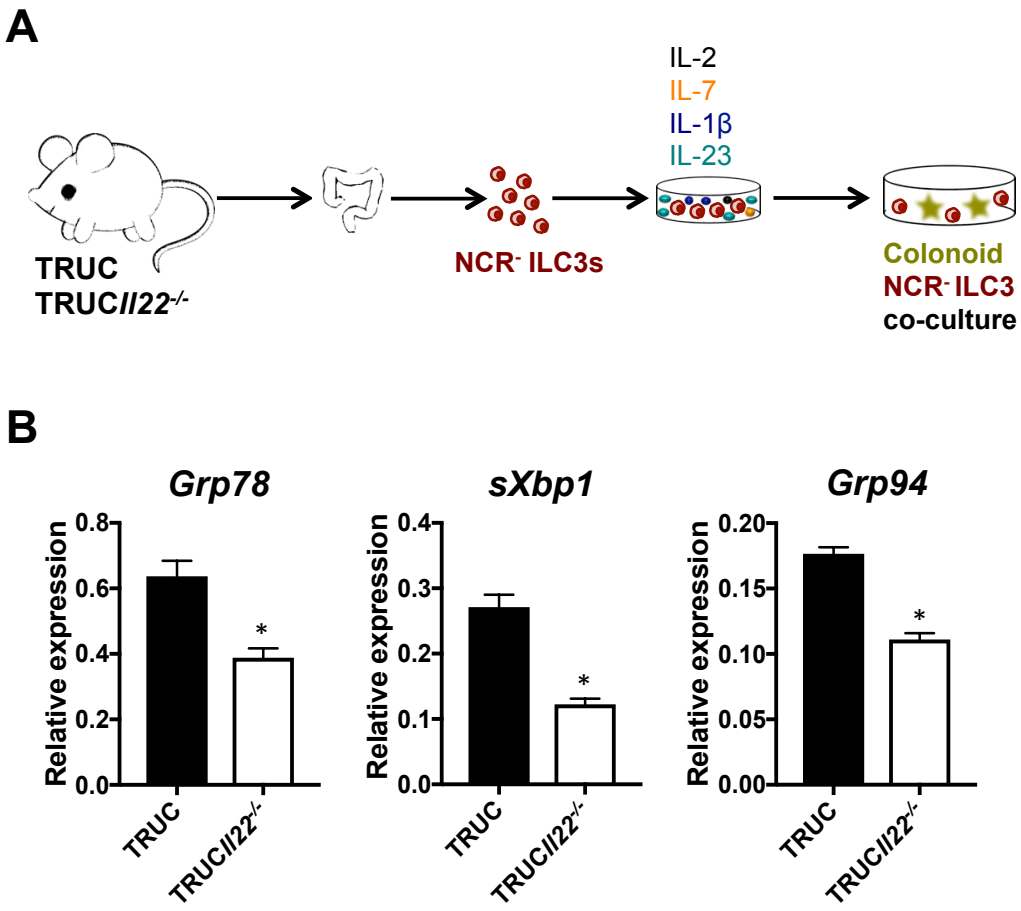

Figure 24: IL-22 sufficient NCR⁻ ILC3s upregulated ER stress genes in colonic epithelial cells. A. NCR⁻ ILC3s were FACS purified from the colon of TRUC and TRUC*Il22*$^{-/-}$ mice, stimulated for 48h with 20ng/ml IL-2, 50ng/ml IL-7, 10ng/ml IL-1β and 10ng/ml IL-23, and then co-cultured for 24h with mouse colonoids. Transcripts of known ER stress genes were quantified by RT-qPCR. B. *Grp78*, *sXbp1* and *Grp94* expression in mouse colonoids co-cultured for 24h with NCR⁻ ILC3s FACS purified from the colon of TRUC and TRUC*Il22*$^{-/-}$ mice, respectively. Data representative of a single experiment with 3 technical replicates. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney one-tailed test, *p<0.05, **P<0.005, ***p<0.0001)

4.4 Inhibition of ER stress with 4-PBA attenuated TRUC disease

To formally address the impact of IL-22 induced ER stress in TRUC disease, TRUC mice were orally administered with the known ER stress inhibitor 4-phenylbutryic acid (4-PBA) (He, Zou et al. 2017) for a period of 6 weeks as shown in Figure 25A. Throughout the course of this experiment mice were regularly checked for weight loss and general signs of distress by Dr Nick Powell. Interestingly, as shown in Figure 25B and 25C, *in vivo* administration of the ER stress inhibitor 4-PBA significantly ameliorated the histological severity of TRUC disease. Moreover, TRUC mice treated with 4-PBA had significantly lighter colons compared to control mice treated with water, but no difference in their spleen weights as depicted in Figure 25D. Taken together these data suggest a direct link between ER stress and TRUC disease.

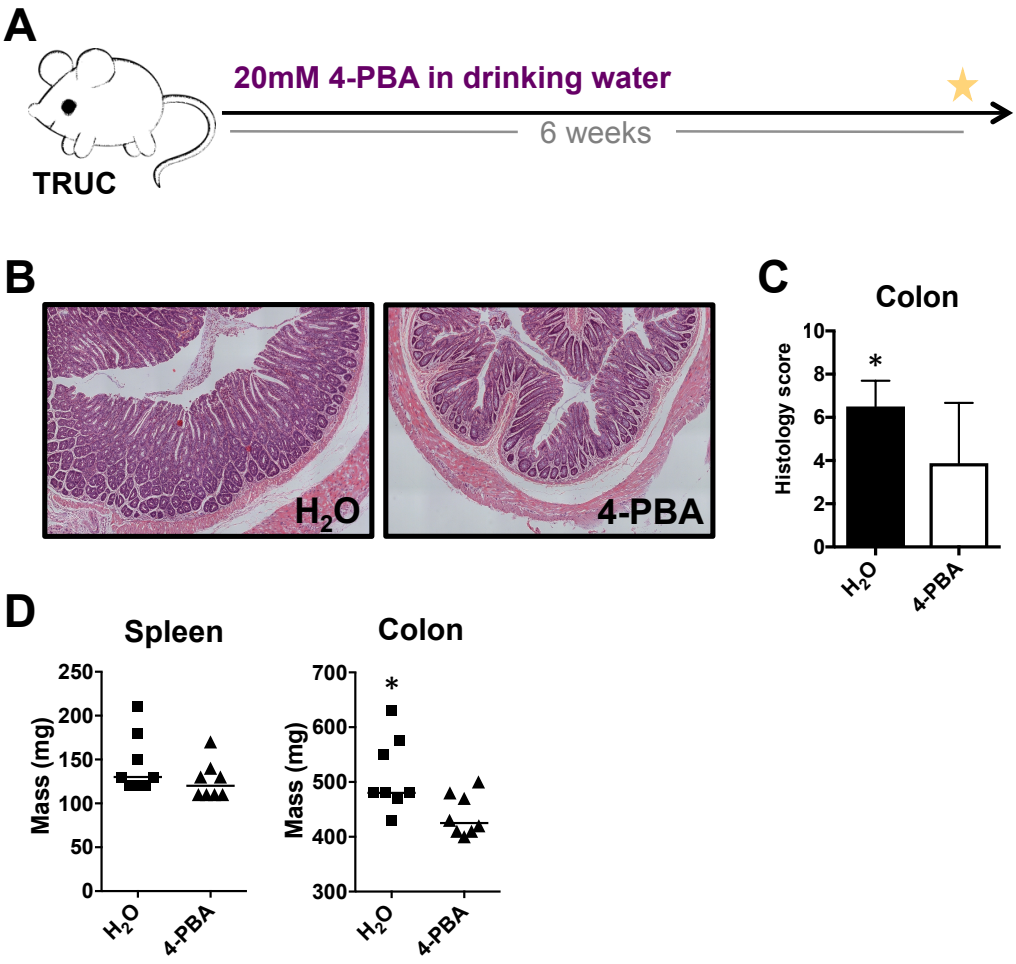

Figure 25: ***In vivo*** **administration of 4-PBA attenuates TRUC disease.** A. 20mM 4-PBA in drinking water was administered to TRUC mice for a period of 6 weeks. B. H&E staining on sections from the distal colon of TRUC mice treated with 20mM 4-PBA or H_2O (controls), respectively. C. Histology score (mean with SD) of TRUC mice treated with 20mM 4-PBA or H_2O (controls), respectively. Data representative of a single experiment with n=8. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney one-tailed test, *p<0.05, **P<0.005, ***p<0.0001). D. Spleen and colon weights (line depicts mean) of TRUC mice treated with 20mM 4-PBA or H_2O (controls), respectively. Each square/triangle represents one mouse. Data representative of a single experiment with n=8. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney one-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

4.5 Induction of ER stress with Tunicamycin induced colitis in otherwise protected TRUC*Il22*$^{-/-}$ mice

Driven by these data, the possibility that restoration of ER stress might rescue the colitis phenotype in otherwise healthy TRUC*Il22*$^{-/-}$ mice was considered. Therefore, the hypothesis that experimental induction of ER stress using Tunicamycin will have an impact on colitis development in TRUC*Il22*$^{-/-}$ mice was tested. For this purpose, TRUC*Il22*$^{-/-}$ mice were intrarectally administered with Tunicamycin by Dr Nick Powell following the experimental protocol shown in Figure 26A. Mice were frequently monitored for weights loss and general signs of distress. As depicted in Figures 26B and 26C, *in vivo* administration of the ER stress inducer Tunicamycin resulted in the development of colitis in TRUC*Il22*$^{-/-}$ mice, which did not occur in control mice administered the vehicle control. In addition, colon weights were also significantly increased in TRUC*Il22*$^{-/-}$ mice treated with Tunicamycin in comparison with control mice (Figure 26D). Taken all together these data show for the first time a role for ER stress in TRUC pathogenesis.

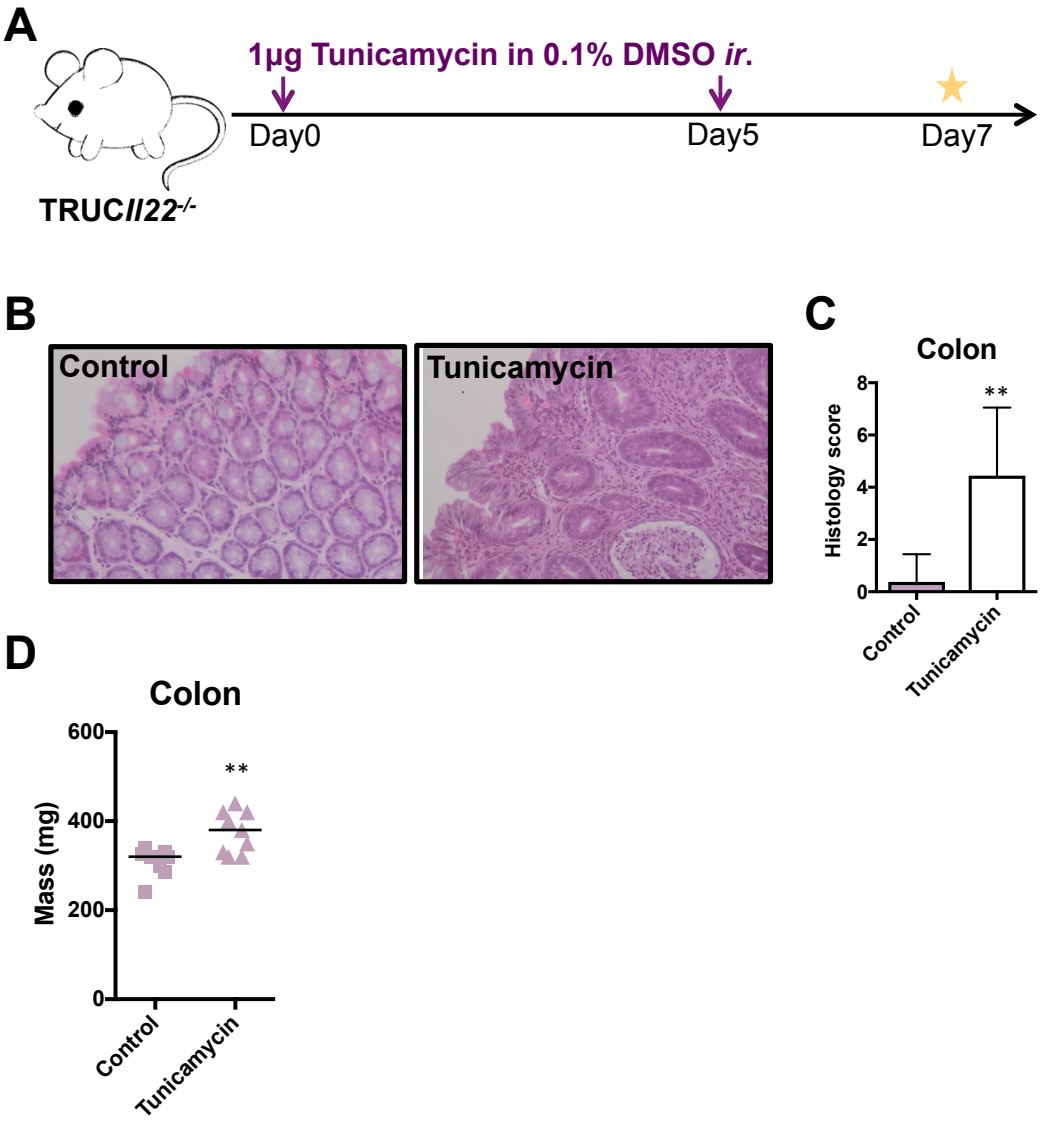

Figure 26: *In vivo* administration of Tunicamycin induces colitis in TRUC*Il22*$^{-/-}$ mice. A. 1µg of Tunicamycin dissolved in 0.1% DMSO (or just 0.1% DMSO) was administered *ir.* to TRUC*Il22*$^{-/-}$ mice at day 0 and day 5, and mice were culled at day 7 for downstream analysis. B. H&E staining on sections from the distal colon of TRUC*Il22*$^{-/-}$ mice treated with or without Tunicamycin, respectively. C. Histology score (mean with SD) of TRUC*Il22*$^{-/-}$ mice treated with or without Tunicamycin respectively. Data representative of a single experiment with n=9 (Tunicamycin) and n=8 (0.1% DMSO). Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney one-tailed test, *p<0.05, **P<0.005, ***p<0.0001). D. Colon weights (line depicts mean) of TRUC*Il22*$^{-/-}$ mice treated with or without Tunicamycin, respectively. Each square/triangle represents one mouse. Data representative of a single experiment with n=9 (Tunicamycin) and n=8 (0.1% DMSO). Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney one-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

4.6 IL-22 and IL-17A synergistically induced expression of ER stress genes in colonic epithelial cells

Since previous work by Dr Nick Powell in our lab has already established a pathogenic role for IL-17A in TRUC disease, and as IL-17A is produced in combination with IL-22 by colonic NCR^- ILC3s, the hypothesis of IL-17A synergizing with IL-22 to induce ER stress was tested. Therefore, and in order to examine whether the effects of IL-22 and potentially IL-17A were the same across the fully differentiated epithelium and the epithelial stem cells, mouse colonoids from *Lgr5*-eGFP reporter mice were generated by our collaborator Dr Anastasia Tsakmaki. As Lgr5 is a known marker for epithelial stem cells, using mouse colonoids generated *Lgr5*-eGFP reporter mice would allow us to test whether IL-22 and potentially IL-17A act solely on the differentiated epithelium to induce ER stress or on the pluripotent epithelial stem (GFP^+) cells as well. Following 24h stimulation with or without IL-22 and IL-17A, single cells suspensions were prepared from the colonoids and *Lgr5*-$eGFP^+$ and *Lgr5*-$eGFP^+$ cells were FACS sorted based on eGFP expression as shown in Figure 27A. To confirm purity, *Lrg5* expression on the two populations was also measured by RT-qPCR as shown in Figure 27B by Dr Anastasia Tsakmaki.

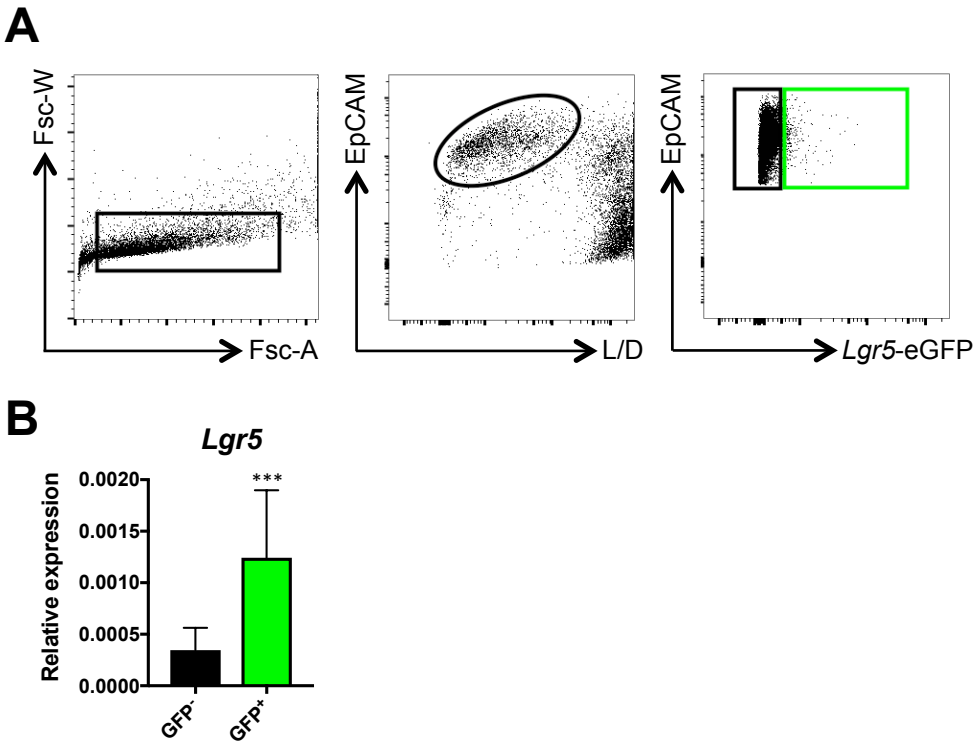

Figure 27: Gating strategy used to FACS purify colonic epithelial cells from mouse colonoids. Mouse colonoids were generated as described previously using *Lgr5*-eGFP mice. Single cell suspensions were prepared and cells were stained with surface markers to allow FACS purification of epithelial ($EpCAM^+$) cells. Epithelial stem cells ($EpCaM^+Lgr5^+$) were identified as GFP^+ cells. A. Flow plots showing gating strategy used to FACS sort epithelial cells and epithelial stem cells from mouse colonoids. Data representative of two independent experiments with n=3. Data were analysed using FlowJo software (Treestar). B. *Lgr5* expression measured by RT-qPCR on epithelial cells and epithelial stem cells sorted from mouse colonoids. Data representative of two independent experiments with n=3. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney one-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

Moreover, FACS analysis was also performed on the sorted populations to examine whether or not there are any differences in the expression of IL-22R and IL-17R between colonic epithelial and colonic epithelial stem cells. As shown in Figure 28, it seems that epithelial stem cells express higher levels of both IL-22R and IL-17R than epithelial cells, suggesting that IL-22 and possibly IL-17A may have a greater impact on epithelial stem cells than on the fully differentiated epithelium.

Finally, as shown in Figure 29, IL-22 and IL-17A stimulation significantly increased the expression of both *Grp78* and *sXbp1* in both colonic epithelial and colonic epithelial stem cells, although the induction in the *Lgr5*$^{+}$ population was greater. Together these data suggest that IL-17A synergizes with IL-22 to induce ER stress in colonic epithelial cells, and in particular in the colonic stem cells supporting even further the notion of NCR^{-} ILC3s being the key drivers of colitis in TRUC disease.

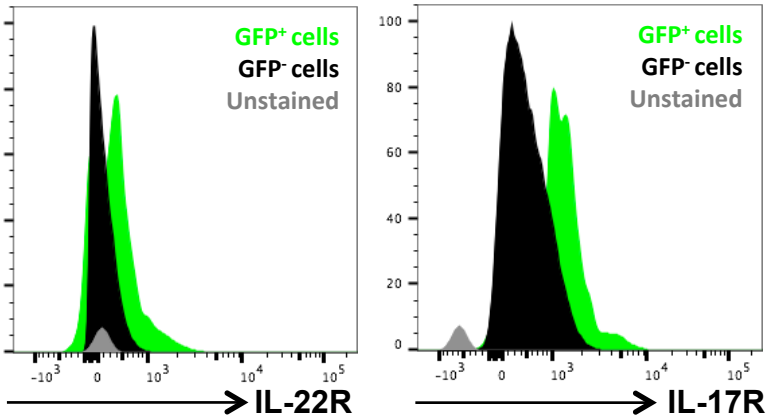

Figure 28: IL-22R and IL-17R expression on colonic epithelial (GFP^-) and colonic stem cells (GFP^+). Mouse colonoids were generated as described previously using *Lgr5*-eGFP mice. Single cell suspensions were prepared and cells were stained with surface markers to allow FACS purification of epithelial ($EpCAM^+$) cells. Epithelial stem cells ($EpCaM^+Lgr5^+$) were identified as GFP^+ cells. Histogram overlays showing IL-22R and IL-17R expression respectively on epithelial (GFP^-) and epithelial stem (GFP^+) cells from mouse colonoids. Data representative of two independent experiments with n=3. Data were analysed using FlowJo software (Treestar).

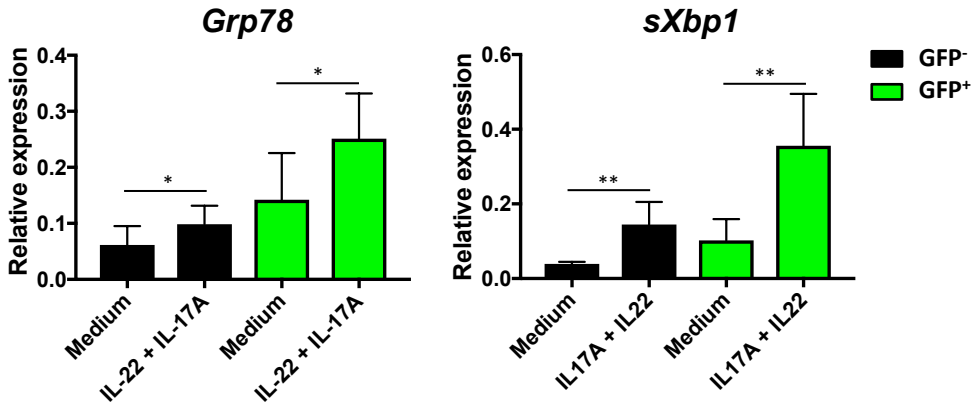

Figure 29: *Grp78 and sXbp1* expression on colonic epithelial (GFP$^-$) and colonic stem cells (GFP$^+$). Mouse colonoids were generated as described previously using *Lgr5*-eGFP mice and stimulated for 24h with our without 10ng/ml IL-22 and 50ng/ml IL-17A. Single cell suspensions were prepared and cells were stained with surface markers to allow FACS purification of epithelial (EpCAM$^+$) cells. Epithelial stem cells (EpCaM$^+$Lgr5$^+$) were identified as GFP$^+$ cells. Graphs showing *Grp78* and *sXbp1* expression (mean with SD) on epithelial cells (GFP$^-$) and epithelial stem cells (GFP$^+$) FACS sorted from mouse colonoids treated with or without 10ng/ml IL-22 and 50ng/ml IL-17A. Data representative of two independent experiments with n=3. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney one-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

4.7 Impact of regulatory T-cells on IL-22 induced epithelial ER stress

The final question tested in this chapter, was whether this profound effect of IL-22 on the colonic epithelium could be in any way regulated. Although the data generated in this section are preliminary, and further work is needed to verify or challenge the results generated, they have been included in this thesis as interesting preliminary findings. In order to generate a more physiological setting [since TRUC mice do not have regulatory T cells (Tregs)], Tregs from the spleen of FoxP3-eGFP reporter mice were FACS sorted as shown in Figure 30A, mixed with aCD3 beads and added to colonoid cultures for a period of 24h in the presence or absence of the ER stress inducing IL-22. To determine whether any of the changes observed might be dependent on the immunosuppressive cytokine IL-10, which has been shown previously to alleviate ER stress (Shkoda, Ruiz et al. 2007), neutralizing anti-IL-10 mAbs were also used in some conditions. Interestingly, as depicted in Figure 30B, Tregs significantly reduced IL-22 induced ER stress in an IL-10 independent manner.

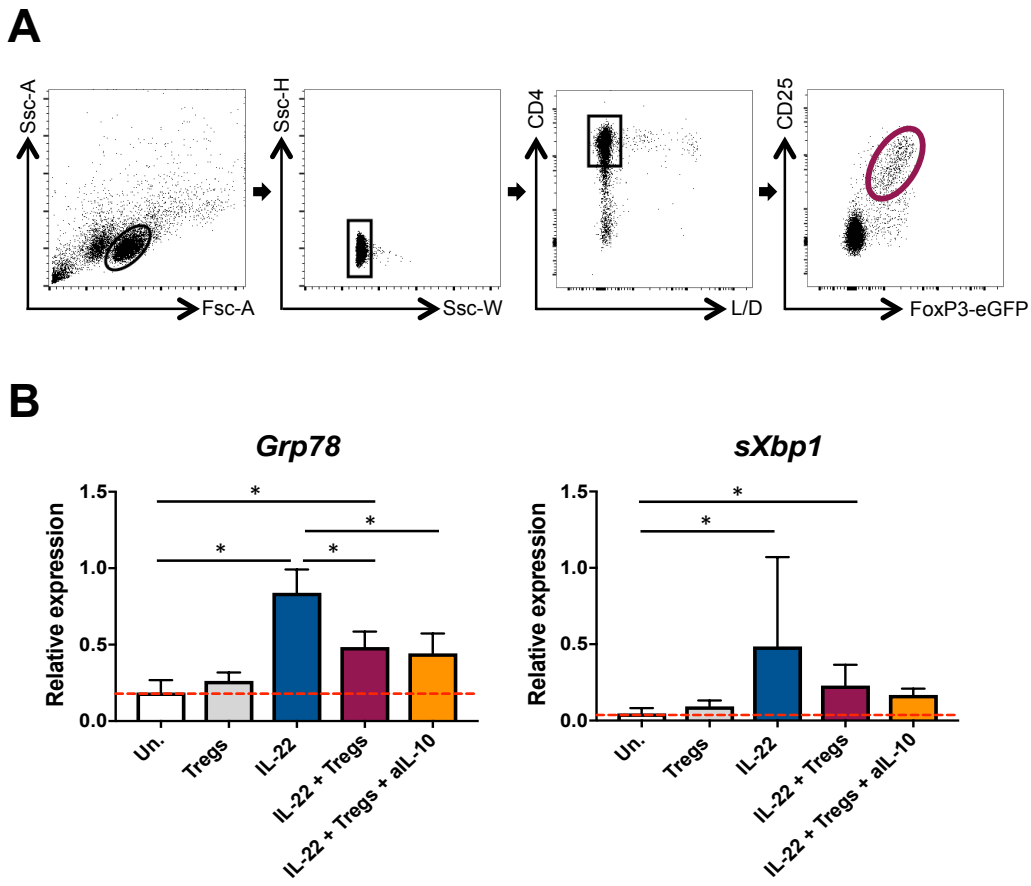

Figure 30: Regulatory T cells significantly reduce IL-22 induced ER stress in colonic epithelial cells. Mouse colonoids were generated as described previously. FoxP3^{+} Tregs were FACS purified from the spleen of *FoxP3*-eGFP reporter mice, mixed with aCD3 beads in 1:2 ratio and co-cultured for 24h with mouse colonoids in the presence or absence of 10ng/ml IL-22. A. Gating strategy used to FACS sort regulatory T cells from the spleen of *FoxP3*-eGFP reporter mice. Data were analysed using FlowJo software (Treestar). B. *Grp78* and *sXbp1* expression (mean with SD) in colonoids co-cultured with Tregs for 24h in the presence or absence of IL-22 with or without aIL-10. Data representative of a single experiment with 6 technical replicates. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney one-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

To confirm this surprising finding, another co-culture experiment was performed with Tregs FACS sorted from the spleens of WT and *Il10*$^{-/-}$ mice as shown in Figure 31A. However, although at a glance it may seem that indeed Tregs can supress ER stress induction by IL-22 in an IL-10 independent manner, in this experiment IL-22 alone failed to significantly increase ER stress in the treated colonoids as shown in Figure 31B making these data un-interpretable.

The same problem was encountered when colonoids were treated for 24h with IL-22 in the presence or absence of IL-10 (Figure 32), magnifying the need for further experiments in order to establish whether or not Tregs supress IL-22 induced ER stress in the colonic epithelial cells via the IL-10 pathway.

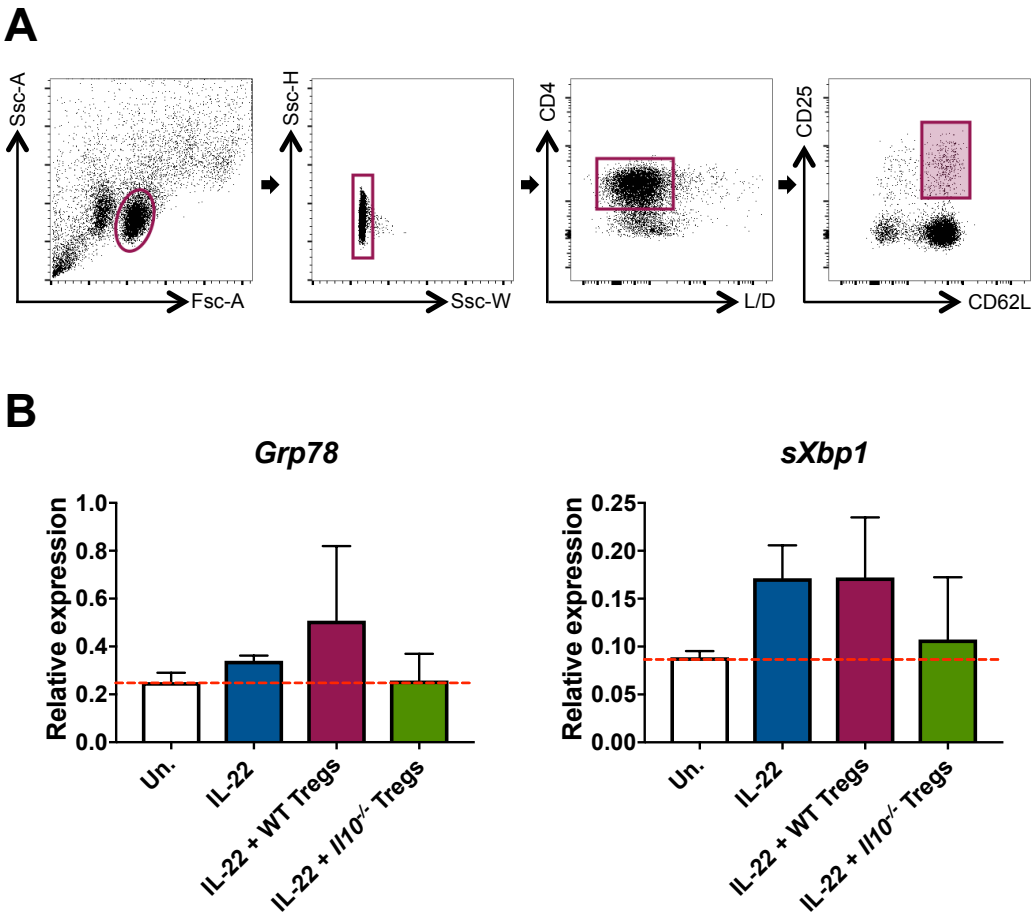

Figure 31: Regulatory T cells seem to reduce IL-22 induced ER stress in colonic epithelial cells. Mouse colonoids were generated as described previously. Tregs were FACS purified from the spleen of WT and *Il10*$^{-/-}$ mice, mixed with aCD3 beads in 1:2 ratio and co-cultured for 24h with mouse colonoids in the presence or absence of 10ng/ml IL-22. A. Gating strategy used to FACS sort regulatory T cells from the spleen of WT and *Il10*$^{-/-}$ mice. Data were analysed using FlowJo software (Treestar). B. *Grp78* and *sXbp1* expression (mean with SD) in colonoids co-cultured with WT or *Il10*$^{-/-}$ Tregs for 24h in the presence or absence of IL-22. Data representative of a single experiment with 6 technical replicates. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney one-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

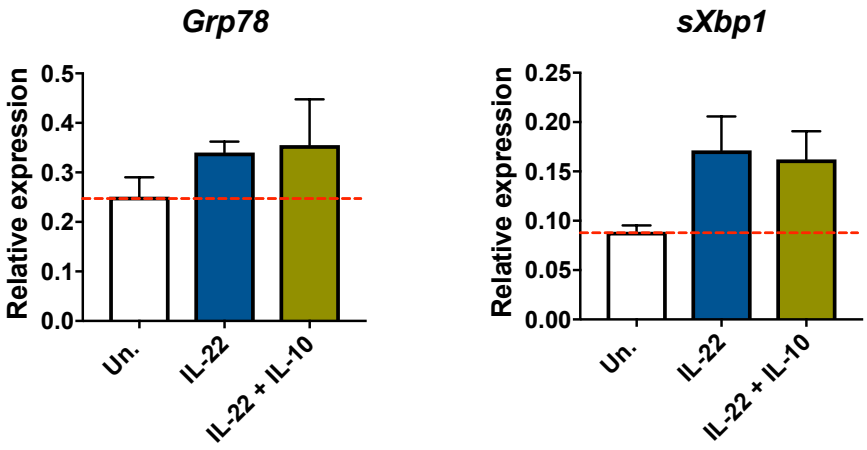

Figure 32: Effect of IL-10 on IL-22 induced ER stress in colonic epithelial cells. Mouse colonoids were generated as described previously and stimulated for 24h with or without 10ng/ml IL-22 in the presence or absence of IL-10. Graphs showing *Grp78* and *sXbp1* expression (mean with SD) in colonoids stimulated for 24h with or without IL-22 in the presence or absence of IL-10. Data representative of a single experiment with 3 technical replicates. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney one-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

4.8 Discussion

In chapter 3 it was shown that IL-22 producing NCR$^-$ ILC3s drive disease in TRUC mice. The data shown in this chapter (4) suggest that IL-22 producing NCR$^-$ ILC3s might contribute to colonic inflammation in TRUC disease through induction of ER stress in colonic epithelial cells, supporting further the link between ER stress and IBD pathogenesis.

IL-22 stimulation induced transcriptional changes on colonic epithelial cells that were similar to those observed by Tunicamycin- a powerful ER stress inducer (Das, Png et al. 2013). Transcripts for known ER stress genes such as *Grp78* and *sXbp1* were also found increased in the colon of TRUC mice compared to the healthy colon of *Rag2*$^{-/-}$ control mice. These findings were also confirmed by RT-qPCR kindly performed by Dr Anastasia Tsakmaki, suggesting that ER stress may be involved in TRUC pathology. A better, un-bias approach to define whether ER stress could potentially be associated with TRUC disease would be to perform pathway analysis on the gene sets generated by whole transcriptome analysis on the colon of TRUC vs. *Rag2*$^{-/-}$ control mice. Interestingly, pathway analysis on genes induced on colonic epithelial cells following IL-22 stimulation showed that indeed ER stress and apoptosis related genes were upregulated in colonic epithelial cells by IL-22. Additional GSEA could reveal exactly how much the ER stress pathway is enriched in the colon of TRUC mice. However, induction of known ER stress genes by IL-22 is not necessarily indicative of ER stress presence in these cells. Further experiments including additional and possibly better read-outs for ER stress are needed to corroborate these findings and firmly link ER stress to TRUC pathology.

A co-culture experiment of FACS purified NCR$^-$ ILC3s isolated from TRUC and TRUC*Il22*$^{-/-}$ mice with murine colonoids revealed that only IL-22 sufficient NCR$^-$ ILC3s were able to significantly induced the expression of the ER stress genes *Grp78*, *sXbp1* and *Grp94* in colonic epithelial cells. These findings further support the notion of IL-22 producing NCR$^-$ ILC3s being the key inducers of ER stress in colonic epithelial cells. Both *Grp78* and *sXbp1* transcripts have been found increased in the small intestine and colon of patients with CD and UC compared to healthy individuals (Kaser, Lee et al. 2008, Shkoda, Ruiz et al. 2007, Deuring, de Haar et al. 2012) highlighting the importance of these molecules in the development of

intestinal inflammation. Moreover, mice in which *Xbp1* is genetically disrupted and thus are unable to control ER stress have been shown to be more susceptible to intestinal inflammation (Kaser, Lee et al. 2008).

In accordance with ER stress being involved in TRUC pathology, in this chapter it was also shown that *in vivo* administration of the known ER stress inhibitor 4-PBA (He, Zou et al. 2017) attenuated TRUC disease. TRUC mice treated with 4-PBA had significantly lighter colons and lower histological scores. Moreover, *in vivo* administration of the ER stress inducer Tunicamycin was able to induce colitis to otherwise healthy TRUC*Il22*$^{-/-}$ mice. Although these experiments were only performed once and need to be repeated, they showed for the first time a clear involvement of ER stress in the pathology of TRUC disease. These findings support further the current notion of ER stress being involved in IBD pathogenesis. Furthermore, additional work in the lab revealed that *in vivo* blockade of IL-22 not only attenuated TRUC disease, but also reduced the expression of known ER stress genes in the colon of TRUC mice (data not shown). Similarly, in vivo administration of recombinant IL-22 to TRUC*Il22*$^{-/-}$ mice not only induced colonic inflammation, but also increased the expression of known ER stress genes in the colon of these mice as well (data not shown). Taken together these data show for the first time a direct link between ER stress and TRUC disease, and suggest that NCR^{-} ILC3s may contribute to colonic inflammation in TRUC mice through IL-22 mediated induction of ER stress in colonic epithelial cells.

In contrast to NCR^{+} ILC3s that almost exclusively produce IL-22, NCR^{-} ILC3s also secrete IL-17A, a cytokine that has already been found to be a key regulator of intestinal inflammation in TRUC mice (Powell, Walker et al. 2012, Powell, Lo et al. 2015). Interestingly, additional work in the lab showed that IL-17A magnified the induction of ER stress genes observed in IL-22 stimulated colonic epithelial cells (data not shown). Moreover, in this chapter it was shown that IL-22 and IL-17A were able to synergistically induce the expression of known ER stress genes in both colonic epithelial and epithelial stem cells, and notably, this effect was more profound in the later. Taking into account that NCR^{-} ILC3s constitute the majority of ILC3s in the colonic LP, as well as their ability to produce pro-inflammatory cytokines such as IL-17A and IFNγ, the data presented so far suggest additional

ways with which these cells might regulate immunopathology in TRUC mice. Moreover, these findings support the hypothesis of IL-22 having diverse and context-dependent actions in the gut mucosa, where in the presence of other pro-inflammatory signals (e.g. IL-17A) during chronic inflammation IL-22 signaling may promote inflammatory responses rather than tissue repair and regeneration.

At the final section of chapter 4, the hypothesis that regulatory T cells (Tregs) might regulate IL-22 mediated induction of ER stress in colonic epithelial cells was also tested. Preliminary findings suggested that likely Tregs could regulate ER stress induction in colonic epithelial cells in an IL-10 independent way, since Tregs were able to significantly reduce the expression of ER stress genes *Grp79* and *sXbp1* in colonic epithelial cells, while neutralizing antibody against IL-10 was able to reverse this effect. Notably, these findings contradict already published studies describing IL-10 as a potential ER stress inhibitor (Shkoda, Ruiz et al. 2007). However, in follow-up experiments that were performed to validate and confirm these findings IL-22 itself failed to induce the expression of the known ER stress genes *Grp79* and *sXbp1* in colonic epithelial cells, making these data un-interpretable. Therefore, further studies are necessary to scrutinize whether IL-22 mediated induction of ER stress in colonic epithelial cells could be regulated by Tregs, and if so the underlying mechanisms.

In summary, the data described in this chapter provide new insights into TRUC pathogenesis by revealing a clear and direct link between ER stress and TRUC disease. Moreover, taken all together these findings form the hypothesis of NCR^- ILC3s being at least partly the drivers of ER stress induced inflammation in colonic epithelial cells through the production of pro-inflammatory cytokines IL-22 and IL-17A, which seem to act both in the fully differentiated epithelium, as well as the colonic epithelial stem cells. The results described in the first two chapters of this thesis, support further the notion of NCR^- ILC3s being the key drivers of intestinal inflammation in TRUC mice, whilst pointing to a pro-inflammatory role of IL-22 in the context of chronic inflammation challenging the current belief of its protective functions during intestinal infections (Sanos, Bui et al. 2009, Satoh-Takayama, Vosshenrich et al. 2008) or chemically induced epithelial injury (Sugimoto, Ogawa et al. 2008). However, this is largely based on transcriptomic data and *in vitro*

experimental settings, and therefore further studies are necessary to formally address the involvement of NCR^- ILC3s and their effector cytokine IL-22 in ER stress induction in the colonic epithelium. Furthermore, as already discussed above additional experiments are also required to establish the presence of ER stress in the colonic epithelium, as increased expression of some known ER stress genes could only be indicative of ER stress in these cells.

Chapter 5

Results: IL-22 drives neutrophil recruitment via induction of CXCL1 and CXCL5 production by colonic epithelial cells

The data presented in chapter 4 of this thesis showed that ER stress is directly involved in the pathology of TRUC disease, whereas it was also suggested that NCR^- ILC3s may drive colonic inflammation through IL-22 mediated induction of ER stress in the colonic epithelium. Since most cytokines are pleiotropic, the hypothesis that IL-22 might have additional effects on the colonic epithelium was tested in this chapter.

Chemokines are small cytokines, which are well known for their important role in regulating leukocyte trafficking to barrier surfaces. In particular, in the gut mucosa it is thought that chemokines produced by epithelial cells induce the recruitment of immune cells such as monocytes, neutrophils and T cells, and thus contribute to or even regulate the inflammatory response (Zimmerman, Vongsa et al. 2008). In accordance with the above belief, several studies have shown elevated expression levels of pro-inflammatory chemokines in CD and UC patients (Reinecker, Loh et al. 1995, Ina, Kusugami et al. 1997, Huang, Eckmann et al. 1996) suggesting a central role for chemokines and the epithelium in the pathogenesis of IBD.

Neutrophil recruitment to the colonic LP is a well-established feature of TRUC disease (Powell, Walker et al. 2012, Ermann, Staton et al. 2014), thus the hypothesis of IL-22 being implicated in this process via induction of neutrophil chemo-attractants was tested in this chapter.

5.1 Chemokine genes are upregulated in TRUC colon

In order to identify any chemokines that regulate neutrophil trafficking to the colonic tissue, whole tissue fragments (0.5cm long) from the distal colon of TRUC and *Rag2*$^{-/-}$ mice were processed for downstream micro array analysis. As shown in Figure 33, several chemokine genes were significantly upregulated in the colon of TRUC mice compared to control *Rag2*$^{-/-}$ mice, including a striking increase in the gene expression of *Cxcl1* and *Cxcl5*. Interestingly, *Cxcl1* and in particular *Cxcl5* are two chemokines known for their role in neutrophil recruitment.

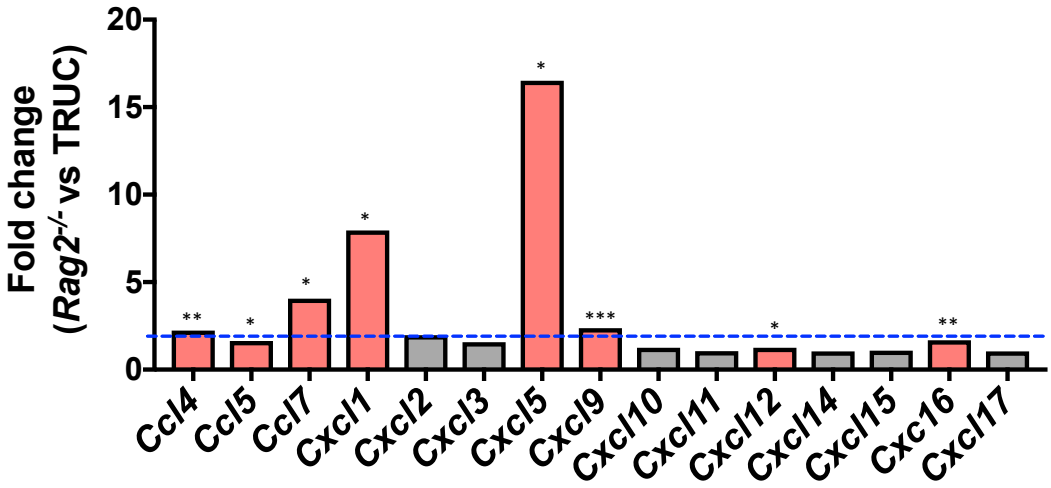

Figure 33: Chemokine gene expression in TRUC mice. 1cm long fragments of distal colon were harvested from 8-12 week old TRUC and *Rag2*$^{-/-}$ (control) mice and whole transcriptome analysis was performed using microarrays (MouseWG-6 v2.0 Expression BeadChip, Illumina). Graph showing fold change of chemokine genes upregulated in the colon of TRUC vs. *Rag2*$^{-/-}$ mice as defined by microarray analysis. Highlighted bars represent significantly upregulated genes. Data representative of a single experiment with 3 biological replicates. Statistical analysis was performed using Partek® and GraphPad PRISM® version 7 software (ANOVA, *p<0.05, **P<0.005, ***p<0.0001).

5.2 IL-22 induces chemokine production in colonic epithelial cells

To interrogate the possibility of IL-22 playing a role in neutrophil recruitment in TRUC disease via induction of CXCL1 and CXCL5 production by colonic epithelial cells, murine colonoids were generated and stimulated for 24h with 10ng/ml recombinant IL-22 by our collaborator Dr Anastasia Tsakmaki for further micro array analysis. As shown in Figure 34, many CXC (Figure 34A) and CC (Figure 34B) chemokine genes were significantly unregulated in the IL-22 treated colonoids compared to unstimulated ones. Most importantly, the same three chemokines (*Ccl7*, *Cxcl1* and *Cxcl5*) that were found significantly upregulated in the TRUC colon, were indeed the most highly upregulated genes in the colonoids upon IL-22 stimulation.

In order to validate the results from the gene array analysis, Dr Anastasia Tsakmaki stimulated again colonoids for 24h with or without 10ng/ml recombinant IL-22 for downstream RT-qPCR analysis. As shown in Figure 35, both *Cxcl1* and *Cxcl5* expression were significantly upregulated in IL-22 treated colonoids compared to controls.

These data were further corroborated in a protein level, as shown in Figure 36. The concentration of both CXCL1 and CXCL5 in the supernatants of IL-22 treated colonoids were significantly higher than those in unstimulated (control) colonoids, as measured by ELISA by Dr Luke Roberts (Figure 36), suggesting that IL-22 may further act on colonic epithelial cells instructing them to produce known neutrophil chemo-attractants such as CXCL1 and CXCL5.

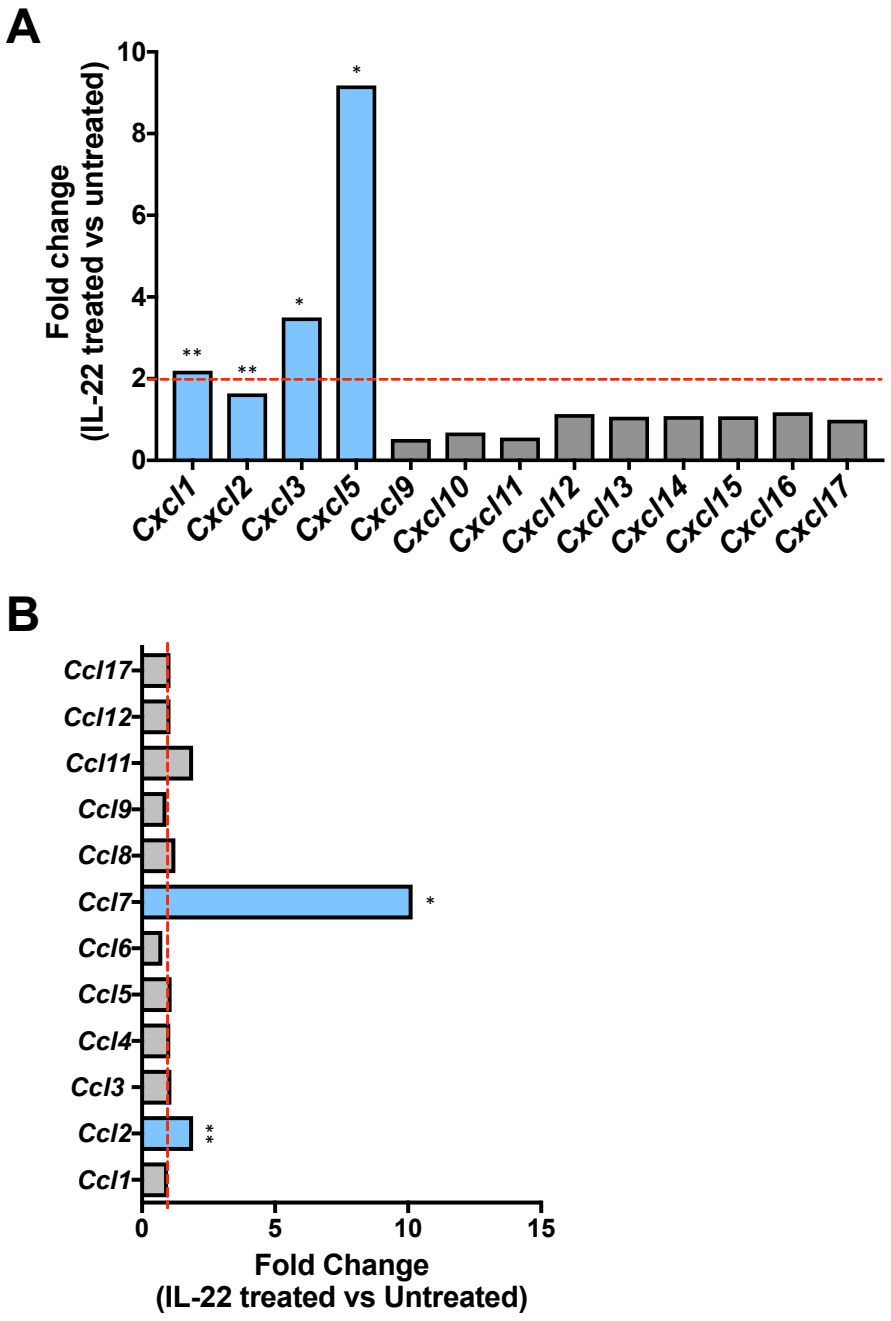

Figure 34: Chemokine gene expression in IL-22 treated colonoids. Mouse colonoids were stimulated for 24h with or without 10ng/ml IL-22 for transcriptome analysis by microarrays (Mouse Gene 2.0 ST array, Affymetrix). A. Graph showing fold change in gene expression of CXC chemokines. Data representative of a single experiment with 3 technical replicates. Blue bars represent significantly differentially expressed transcripts. Data representative of a single experiment with 3 technical replicates. Statistical analysis was performed using Partek® and GraphPad PRISM® version 7 software (ANOVA, *p<0.05, **P<0.005, ***p<0.0001). B. Graph showing fold change in gene expression of Ccl chemokines. Data representative of a single experiment with 3 technical

replicates. Blue bars represent significantly differentially expressed transcripts. Data representative of a single experiment with 3 technical replicates. Statistical analysis was performed using Partek® and GraphPad PRISM® version 7 software (ANOVA, *p<0.05, **P<0.005, ***p<0.0001).

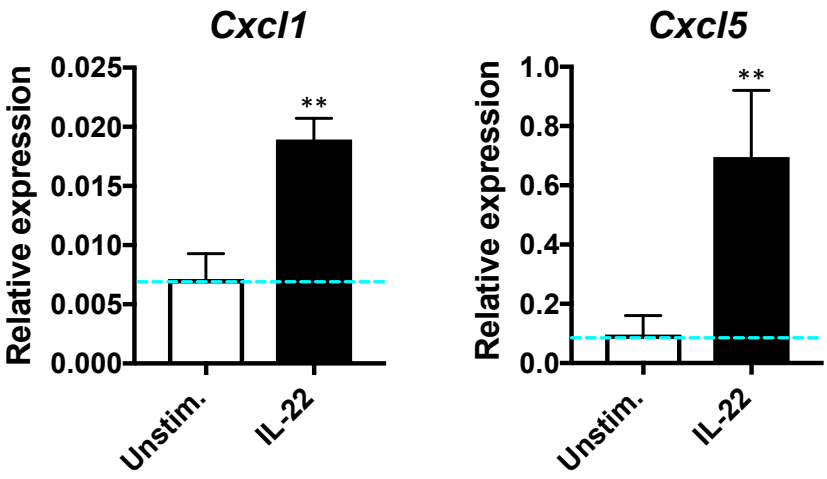

Figure 35: IL-22 induces *Cxcl1* and *Cxcl5* expression in colonic epithelial cells. Mouse colonoids were stimulated for 24h with or without 10ng/ml IL-22 and *Cxcl1* and *Cxcl5* gene expression was measured by RT-qPCR. Graphs showing *Cxcl1* and *Cxcl5* gene expression in mouse colonoids treated with or without IL-22, respectively. Data pooled from two independent experiments with n=3. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney, one-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

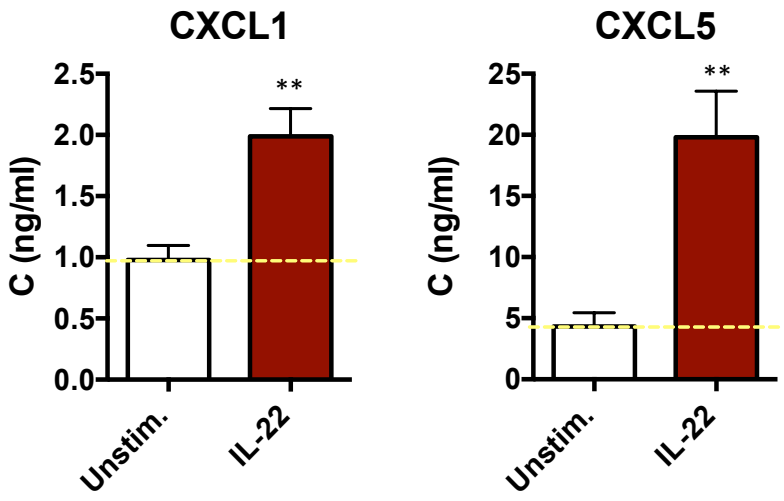

Figure 36: IL-22 induces CXCL1 and CXCL5 production by colonic epithelial cells. Mouse colonoids were stimulated for 24h with or without 10ng/ml IL-22, and CXCL1 and CXCL5 levels in the culture S/Ns were measured by ELISA. Graphs showing the concentration of CXCL1 and CXCL5 in S/Ns of colonoids stimulated with or without IL-22. Data representative of a single experiment with 6 technical replicates. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney, one-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

5.3 IL-22 producing NCR$^-$ ILC3s induce CXCL1 and CXCL5 production by colonic epithelial cells

To formally investigate whether NCR$^-$ ILC3s regulate chemokine production by colonic epithelial cells in an IL-22 dependent manner, NCR$^-$ ILC3s were FACS sorted as described in Chapter 2 from the colon of TRUC and TRUC*Il22*$^{-/-}$ mice respectively, activated through 48h stimulation with the known positive regulators of IL-22, IL-23 and IL-1β, followed by a 24h co-culture with colonoids as shown in Figure 24A. At the end of the 24h stimulation, mRNA levels of *Cxcl1* and *Cxcl5* were measured by RT-qPCR by Dr Anastasia Tsakmaki. As depicted in Figure 37, both *Cxcl1* and *Cxcl5* expression were significantly reduced in colonoids that were co-cultured with IL-22 deficient NCR$^-$ ILC3s compared to the levels in colonoids co-cultured with IL-22 sufficient NCR$^-$ ILC3s. Taken together, this data suggest that NCR$^-$ ILC3s may play a role in the regulation of neutrophil recruitment to the gut through IL-22 mediated induction of CXCL1 and CXCL5 production by colonic epithelial cells.

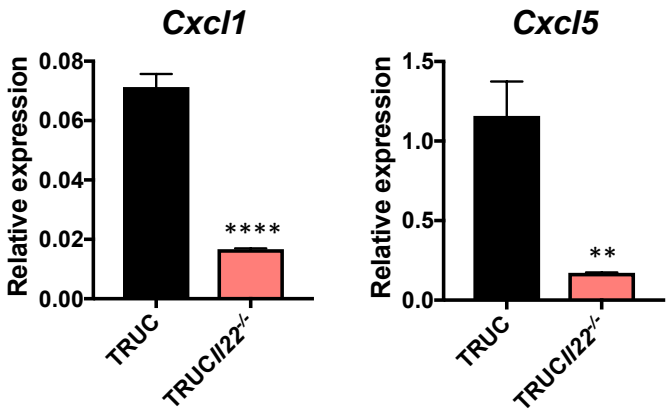

Figure 37: IL-22 sufficient NCR$^-$ ILC3s induce *Cxcl1* and *Cxcl5* expression in colonic epithelial cells. NCR$^-$ ILC3s were FACS purified from the colon of TRUC and TRUC*Il22*$^{-/-}$ mice, stimulated for 48h with 20ng/ml IL-2, 50ng/ml IL-7, 10ng/ml IL-1β and 10ng/ml IL-23, and then co-cultured for 24h with mouse colonoids. *Cxcl1* and *Cxcl5* transcripts were quantified by RT-qPCR. Graphs showing *Cxcl1* and *Cxcl5* expression in mouse colonoids co-cultured for 24h with NCR$^-$ ILC3s FACS purified from the colon of TRUC and TRUC*Il22*$^{-/-}$ mice, respectively. Data representative of a single experiment with 3 technical replicates. Statistical analysis was performed using GraphPad PRISM® version 7 software (Mann-Whitney one-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

5.4 IL-22 drives neutrophil recruitment in TRUC disease

In order to test the hypothesis that in TRUC disease, IL-22 regulates neutrophil recruitment to the cLP by inducing CXCL1 and CXCL5 production by colonic epithelial cells, cLPMCs were isolated from the colon of TRUC and TRUC*Il22*$^{-/-}$ mice and the percentages of neutrophils infiltrating the lamina propria were measured by FACS analysis. As shown in Figures 38A and 38B, the percentage of neutrophils (defined as live CD45^{+}CD11b^{+}Gr-1^{hi} cells) in the cLP of TRUC*Il22*$^{-/-}$ mice is significantly impaired compared to control TRUC mice. However, there were no differences in the absolute number of neutrophils in the colonic LP of TRUC and TRUC*Il22*$^{-/-}$ mice (Figure 38C). Similarly, no differences were found in the total cLPMCs between TRUC and TRUC*Il22*$^{-/-}$ mice (Figure 38D), thus a third repeat of this experiment would most likely help clarify whether genetic deletion of IL-22 is associated with impaired neutrophil recruitment to the gut in TRUC mice.

Consistent with the hypothesis that IL-22 regulates neutrophil accumulation to the cLP of TRUC mice through induction of chemokine production by colonic epithelial cells, TRUC*Il22*$^{-/-}$ mice had significantly reduced percentages of neutrophils in their spleen when compared to control TRUC mice as shown in Figures 39A and 39B. Moreover, the total numbers of neutrophils were also impaired in the spleen of these mice compared to control TRUC mice (Figure 39C). However, there was no difference in the total splenocyte counts between TRUC and TRUC*Il22*$^{-/-}$ mice as shown in Figure 39D.

Interestingly, *in vivo* blockade of IL-22 by *ip.* injections of a neutralizing mAb in TRUC mice, didn't seem to affect the percentage of neutrophils in the cLP of TRUC mice compared to control mice that received the Isotype antibody (Figures 40A and 40B). Yet, the absolute number of neutrophils, as well as the total cLPMCs in the cLP of TRUC mice treated with the aIL-22 mAb were found to be significantly decreased compared to control mice as shown in Figure 40C and 40D, respectively. This experiment supports the hypothesis of IL-22 regulating neutrophil accumulation to the cLP in TRUC disease, but given that it was only performed once, additional repeats are needed to prove this hypothesis.

In an attempt to further explore these findings, recombinant IL-22 was *in vivo* administered *ip.* to TRUC*Il22*$^{-/-}$ mice, and the neutrophils in colon and spleen of these mice were analysed by FACS as previously. As shown in Figures 41A and 41B, the percentage of neutrophils in the cLP of TRUC*Il22*$^{-/-}$ mice that received rIL-22 was significantly increased compared to control mice treated with PBS. In addition, as shown in Figures 41C and 41D, the absolute number of neutrophils as well as the total cLPMCs were also significantly increased in the cLP of rIL-22 treated TRUC*Il22*$^{-/-}$ mice compared to PBS treated mice (controls) respectively, suggesting that indeed IL-22 may be a key regulator of neutrophil recruitment to the gut in TRUC disease.

Surprisingly, *in vivo* administration of rIL-22 significantly reduced the percentage of neutrophils in the spleen of TRUC*Il22*$^{-/-}$ mice compared to PBS treated mice (controls) as shown in Figures 42A and 42B. However, there was no difference in the absolute number of neutrophils in the spleen of TRUC*Il22*$^{-/-}$ mice treated with rIL-22 and PBS treated TRUC*Il22*$^{-/-}$ mice (Figure 42C). Similarly, as shown in Figure 42D, the total number of splenocytes didn't seem to be affected by rIl-22 administration in TRUC*Il22*$^{-/-}$ mice either, suggesting that there may be different mechanisms that regulate neutrophil trafficking to the spleen. Taken all together, these findings suggest that IL-22 may play a role in neutrophil trafficking to the colon during chronic colitis in TRUC mice, possibly through induction of chemokine production such as CXCL1 and CXCL5 by colonic epithelial cells, although additional repeats of these experiments are needed to confirm this hypothesis.

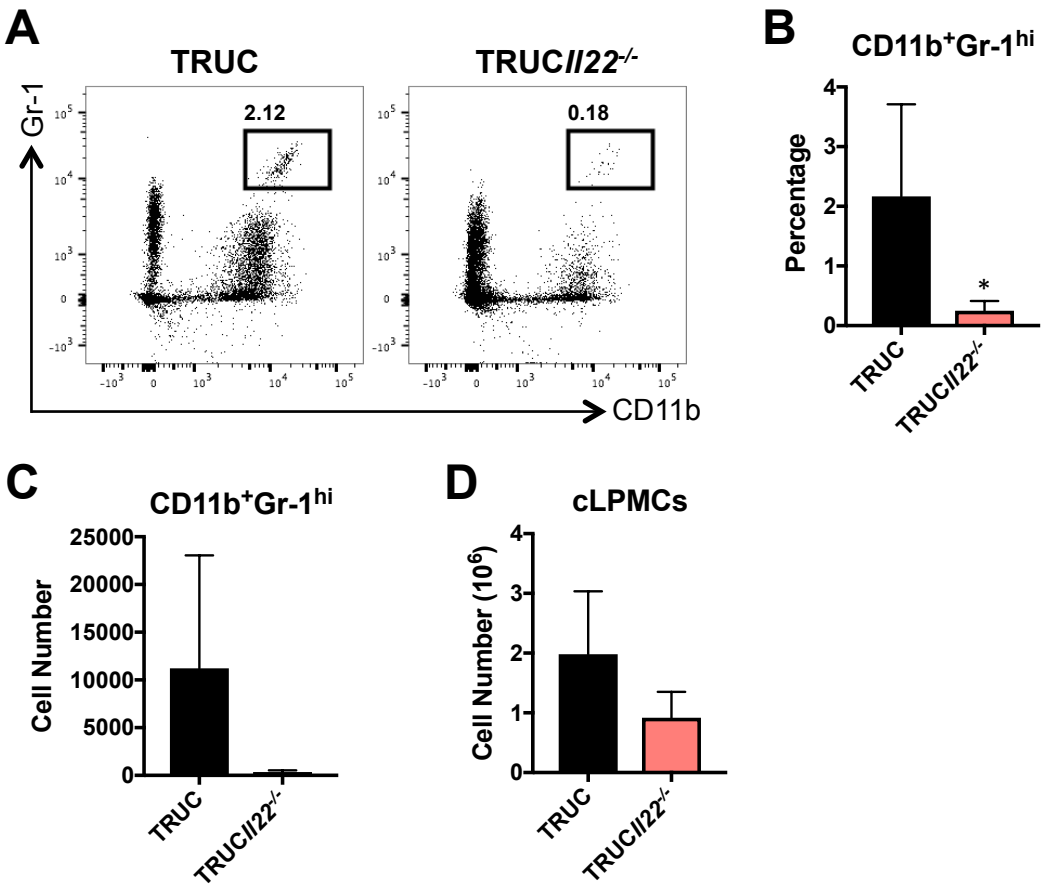

Figure 38: Reduced percentage of neutrophils in the colonic lamina propria of TRUC*Il22*$^{-/-}$ mice. TRUC and TRUC*Il22*$^{-/-}$ mice were orally gavaged with intestinal microbiota at day 0 and culled 6 weeks later at day 42. cLPMCs were isolated using Percoll gradient and stained with Abs against surface markers to analyse neutrophil infiltrate by FACS. A. Representative dot plots showing the percentage of neutrophils defined as CD11b^{+}Gr-1^{hi} cells in the cLP of TRUC and TRUC*Il22*$^{-/-}$ mice, respectively. Cells gated as single live CD45^{+} cells. Data representative of two independent experiments with n=5 and n=6, respectively. B. Graph showing the percentage of CD11b^{+}Gr-1^{hi} cells in the cLP of TRUC and TRUC*Il22*$^{-/-}$ mice. Data representative of two independent experiments with n=5 and n=6, respectively. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). C. Graph showing the absolute number of CD11b^{+}Gr-1^{hi} cells in the cLP of TRUC and TRUC*Il22*$^{-/-}$ mice. Data representative of two independent experiments with n=5 and n=6, respectively. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). D. Graph showing the absolute number of cLPMCs in TRUC vs. TRUC*Il22*$^{-/-}$ mice. Data representative of two independent experiments with n=5 and n=6, respectively. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

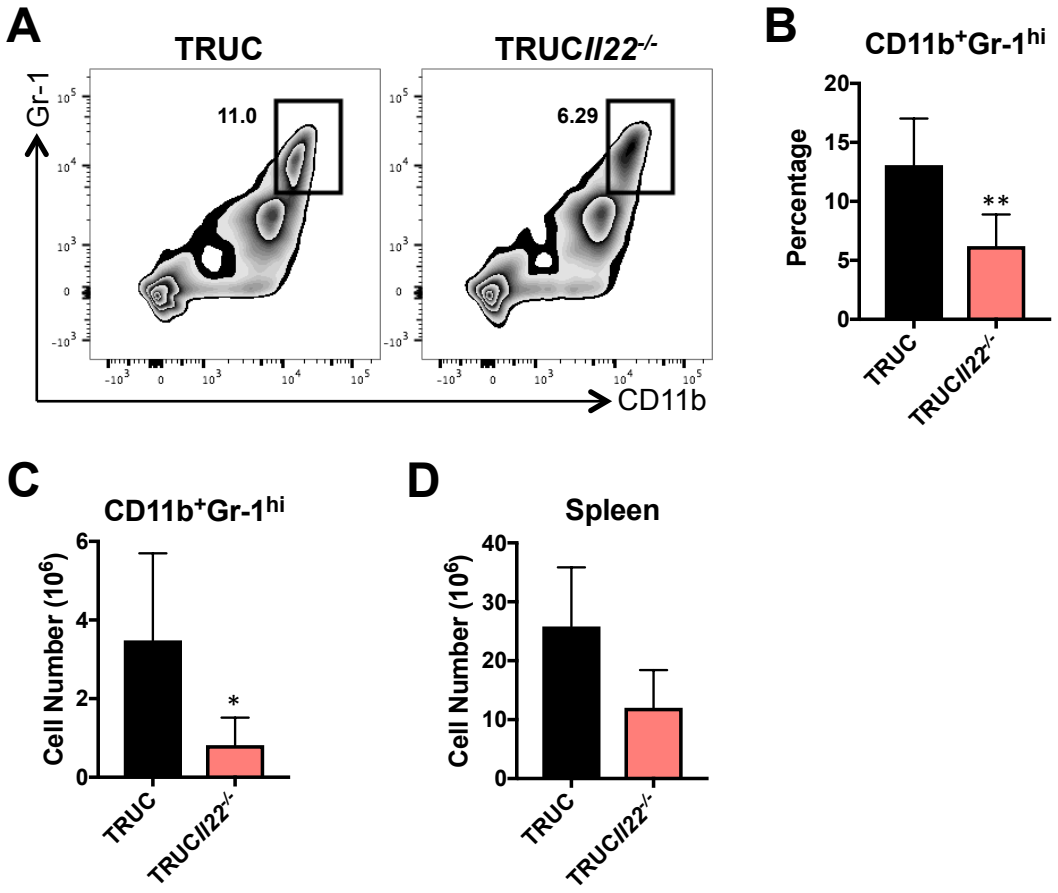

Figure 39: TRUC*Il22*$^{-/-}$ mice exhibit impaired neutrophil recruitment in the spleen. TRUC and TRUC*Il22*$^{-/-}$ mice were orally gavaged with intestinal microbiota at day 0 and culled 6 weeks later at day 42. Single cell suspensions were prepared from the spleens of TRUC and TRUC*Il22*$^{-/-}$ mice and stained with Abs against surface markers to analyse neutrophil infiltrate by FACS. A. Representative dot plots showing the percentage of neutrophils defined as CD11b^{+}Gr-1^{hi} cells in the spleen of TRUC and TRUC*Il22*$^{-/-}$ mice, respectively. Cells gated as single live cells. Data representative of two independent experiments with n=5 and n=6, respectively. B. Graph showing the percentage of CD11b^{+}Gr-1^{hi} cells in the spleen of TRUC and TRUC*Il22*$^{-/-}$ mice. Data representative of two independent experiments with n=5 and n=6, respectively. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). C. Graph showing the absolute number of CD11b^{+}Gr-1^{hi} cells in the spleen of TRUC and TRUC*Il22*$^{-/-}$ mice. Data representative of two independent experiments with n=5 and n=6, respectively. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). D. Graph showing the absolute number of splenocytes in TRUC vs. TRUC*Il22*$^{-/-}$ mice. Data representative of two independent experiments with n=5 and n=6, respectively. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

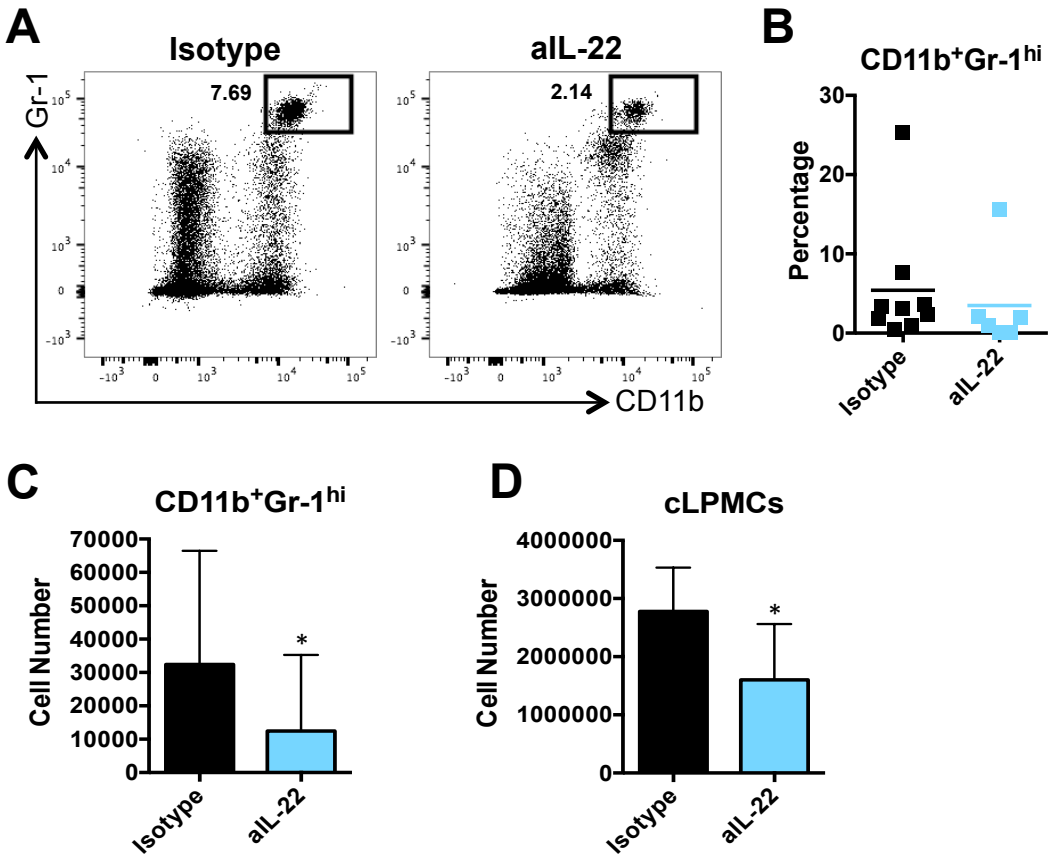

Figure 40: IL-22 blockade resulted in impaired neutrophil numbers in the cLP of TRUC mice. TRUC mice were administered *ip.* with 200µg aIL-22 or isotype antibody (control) and culled seven days later for downstream analysis. cLPMCs were isolated using Percoll gradient and stained with Abs against surface markers to analyse neutrophil infiltrate by FACS. A. Representative dot plots showing the percentage of neutrophils defined as $CD11b^{+}Gr\text{-}1^{hi}$ cells in the cLP of TRUC mice treated with 200µg of aIL-22 (n=6) or isotype control Ab (n=8), respectively. Cells gated as single live CD45+ cells. Data representative of a single experiment. B. Graph showing the percentage of $CD11b^{+}Gr\text{-}1^{hi}$ cells in the cLP of TRUC mice treated with 200µg of aIL-22 (n=6) or isotype control Ab (n=8), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). C. Graph showing the absolute number of $CD11b^{+}Gr\text{-}1^{hi}$ cells in the cLP of TRUC mice treated with 200µg of aIL-22 (n=6) or isotype control Ab (n=8), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). D. Graph showing the absolute number of cLPMCs in the cLP of TRUC mice treated with 200µg of aIL-22 (n=6) or isotype control Ab (n=8), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

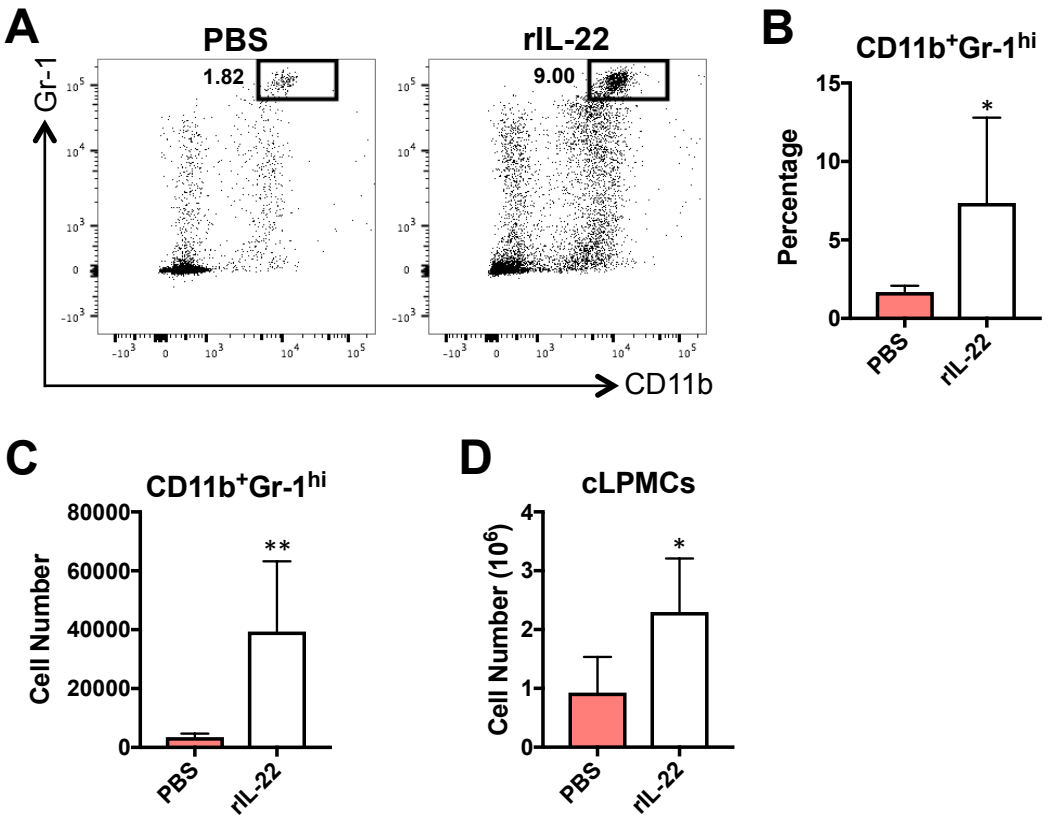

Figure 41: Administration of recombinant IL-22 resulted in increased neutrophil recruitment in the cLP of TRUC*Il22*$^{-/-}$ mice. TRUC*Il22*$^{-/-}$ mice were administered *ip.* with 100µg rIL-22 or PBS (control) at days 0, 4, 8 and 12, and were culled at day 14 for downstream analysis. cLPMCs were isolated using Percoll gradient and stained with Abs against surface markers to analyse neutrophil infiltrate by FACS. A. Representative dot plots showing the percentage of neutrophils defined as CD11b^{+}Gr-1^{hi} cells in the cLP of TRUC*Il22*$^{-/-}$ mice treated with 100µg rIL-22 (n=5) or PBS (control) (n=7), respectively. Cells gated as single live CD45^{+} cells. Data representative of a single experiment. B. Graph showing the percentage of CD11b^{+}Gr-1^{hi} cells in the cLP of TRUC*Il22*$^{-/-}$ mice treated with 100µg rIL-22 (n=5) or PBS (control) (n=7), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). C. Graph showing the absolute number of CD11b^{+}Gr-1^{hi} cells in the cLP of TRUC*Il22*$^{-/-}$ mice treated with 100µg rIL-22 (n=5) or PBS (control) (n=7), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). D. Graph showing the absolute number of cLPMCs in TRUC*Il22*$^{-/-}$ mice treated with 100µg rIL-22 (n=5) or PBS (control) (n=7), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

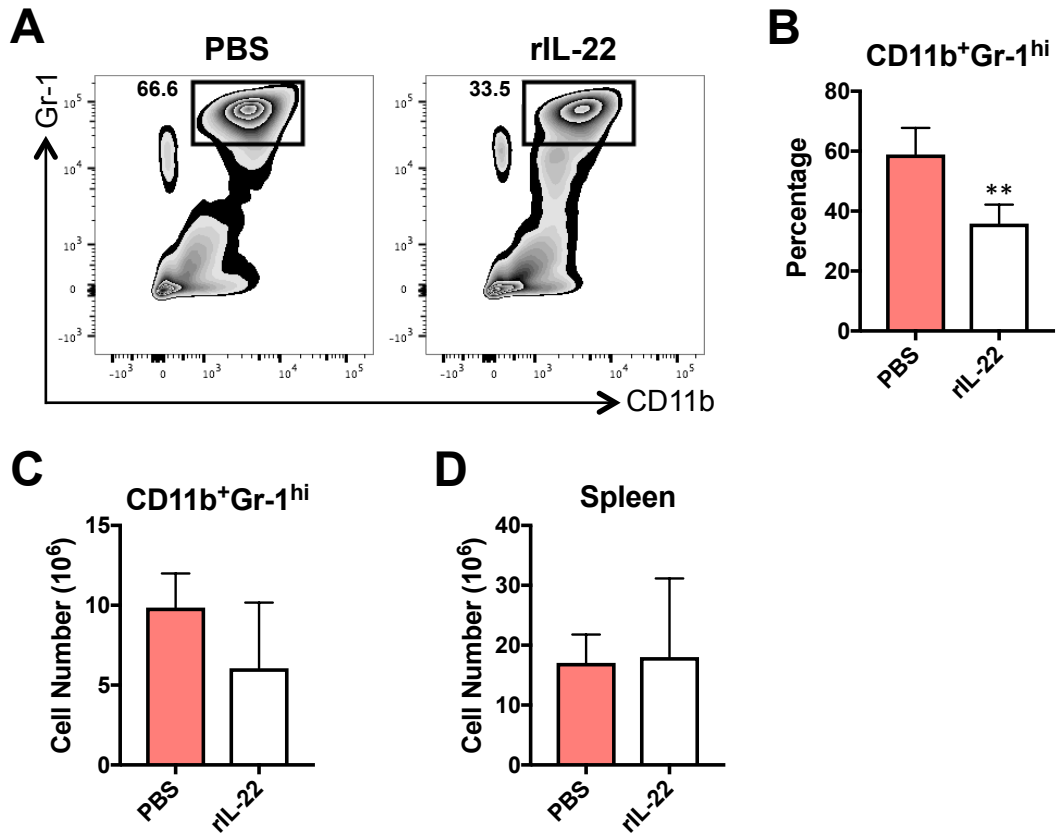

Figure 42: Administration of recombinant IL-22 resulted in increased neutrophil percentages in the spleen of TRUC*Il22*$^{-/-}$ mice. TRUC*Il22*$^{-/-}$ mice were administered *ip.* with 100µg rIL-22 or PBS (control) at days 0, 4, 8 and 12, and were culled at day 14 for downstream analysis. Single cell suspensions were prepared from the spleens of TRUC*Il22*$^{-/-}$ mice treated with 100µg rIL-22 (n=5) or PBS (control) (n=7) and stained with Abs against surface markers to analyse neutrophil infiltrate by FACS. A. Representative dot plots showing the percentage of neutrophils defined as CD11b^{+}Gr-1^{hi} cells in the spleen of TRUC*Il22*$^{-/-}$ mice treated with 100µg rIL-22 (n=5) or PBS (control) (n=7), respectively. Cells gated as single live cells. Data representative of a single experiment. B. Graph showing the percentage of CD11b^{+}Gr-1^{hi} cells in the spleen of TRUC*Il22*$^{-/-}$ mice treated with 100µg rIL-22 (n=5) or PBS (control) (n=7), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). C. Graph showing the absolute number of CD11b^{+}Gr-1^{hi} cells in the spleen of TRUC*Il22*$^{-/-}$ mice treated with 100µg rIL-22 (n=5) or PBS (control) (n=7), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). D. Graph showing the absolute number of splenocytes in TRUC*Il22*$^{-/-}$ mice treated with 100µg rIL-22 (n=5) or PBS (control) (n=7), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

5.5 *In vivo* blocking of CXCL1 didn't seem to affect neutrophil recruitment to the colon of TRUC mice

To formally address a potential involvement of the chemokine CXCL1 that was found to be induced in colonic epithelial cells upon IL-22 stimulation, in neutrophil accumulation to the cLP of TRUC mice, a neutralizing mAb against CXCL1 was *in vivo* administered to TRUC mice, and neutrophil recruitment to the colon of these mice was analysed by flow cytometry. As shown in Figures 43A and 43B, there was no difference in the percentage of neutrophils in the cLP of aCXCL1 treated TRUC mice compared to isotype treated (control) mice. Similarly, there were no differences in the total neutrophil counts or the total cLPMCs between aCXCL1 and isotype treated control mice (Figure 43C and 43D, respectively). Although these findings seem to suggest that CXCL1 is not needed for neutrophil recruitment to the colon of TRUC mice, this experiment was only performed once, hence additional repeats are needed in order to validate the data shown in Figure 43.

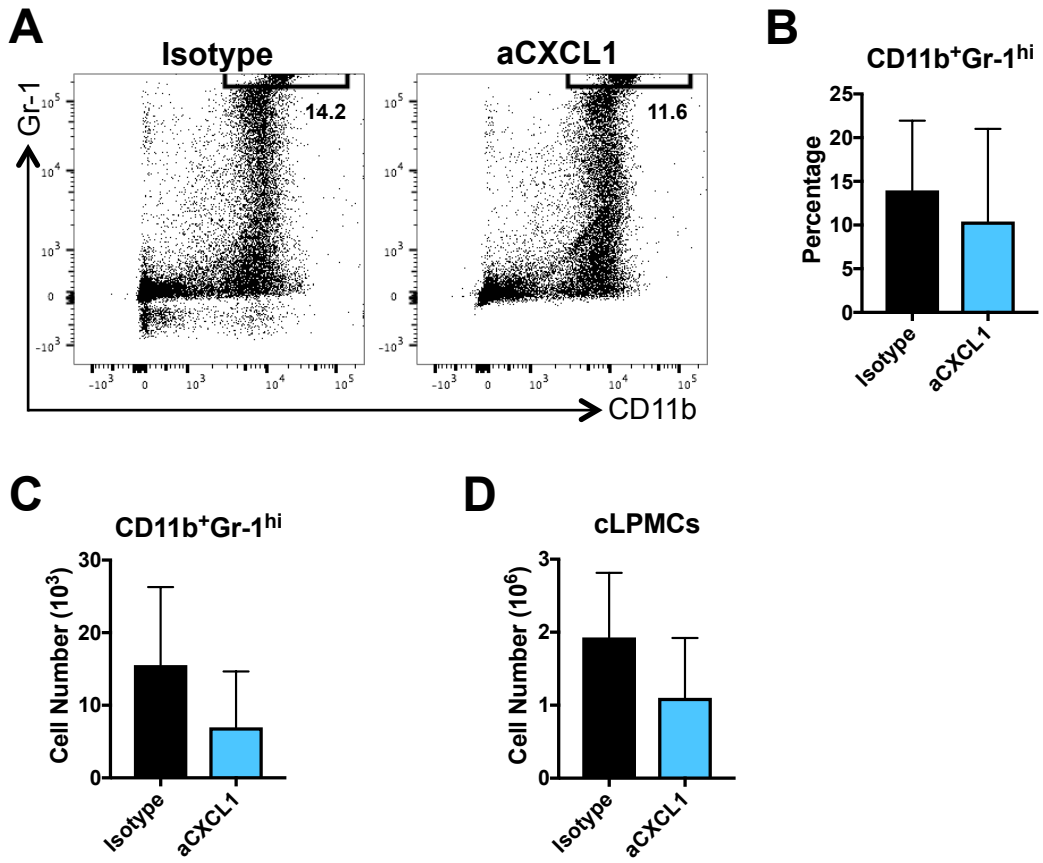

Figure 43: CXCL1 didn't seem to affect neutrophil recruitment to the cLP of TRUC mice. TRUC mice were administered *ip.* with 150µg aCXCL1or isotype antibody (control) every 3 to 4 days and culled for downstream analysis. cLPMCs were isolated using Percoll gradient and stained with Abs against surface markers to analyse neutrophil infiltrate by FACS. A. Representative dot plots showing the percentage of neutrophils defined as $CD11b^{+}Gr\text{-}1^{hi}$ cells in the cLP of TRUC mice treated with 150µg of aCXCL1 (n=5) or isotype control Ab (n=7), respectively. Cells gated as single live $CD45^{+}$ cells. Data representative of a single experiment. B. Graph showing the percentage of $CD11b^{+}Gr\text{-}1^{hi}$ cells in the cLP of TRUC mice treated with 150µg of aCXCL1 (n=5) or isotype control Ab (n=7), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). C. Graph showing the absolute number of $CD11b^{+}Gr\text{-}1^{hi}$ cells in the cLP of TRUC mice treated with 150µ g of aCXCL1 (n=5) or isotype control Ab (n=7), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). D. Graph showing the absolute number of cLPMCs in TRUC mice treated with 150µ of aCXCL1 (n=5) or isotype control Ab (n=7), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

5.6 *In vivo* blocking of CXCR2 didn't seem to affect neutrophil recruitment to the colon of TRUC mice

Since IL-22 was found to induce both CXCL1 and CXCL5 production by colonic epithelial cells, the effect of *in vivo* blocking both of these two chemokines in neutrophil trafficking in TRUC mice was investigated. Indeed, the failure of CXCL1 blockade alone could be accounted for by its redundancy in this system, with CXCL5 being able to compensate to mediate neutrophil recruitment. Therefore, we sought to block both CXCL1 and CXCL5 by administering a blocking anti-CXCR2 mAb to TRUC mice, since both CXCL1 and CXCL5 use the CXCR2 receptor. As shown in Figures 44A and 44B, CXCR2 blockade didn't affect the percentage of neutrophils in the cLP of TRUC mice. Similarly, the absolute number of neutrophils was also not affected in the cLP of aCXCR2 treated TRUC mice in comparison to isotype treated (control) mice (Figure 44C). *In vivo* blocking of CXCR2 in TRUC mice didn't seem to affect the total cLPMCs either as shown in Figure 44D.

Similarly, *in vivo* administration of CXCR2 didn't affect neutrophil accumulation to the spleen of treated TRUC mice compared to control mice (Figure 45). As shown in Figures 45A and 45B, the percentage of neutrophils in the spleen of aCXCR2 treated TRUC mice is slightly increased if anything, compared to the one in control mice. The absolute neutrophil number, as well as the total splenocyte counts were also unaffected in aCXCR2 treated mice compared to isotype treated (control) mice as shown in Figures 45C and 45D, respectively. Again, additional repeats of these experiments are needed to confirm these findings.

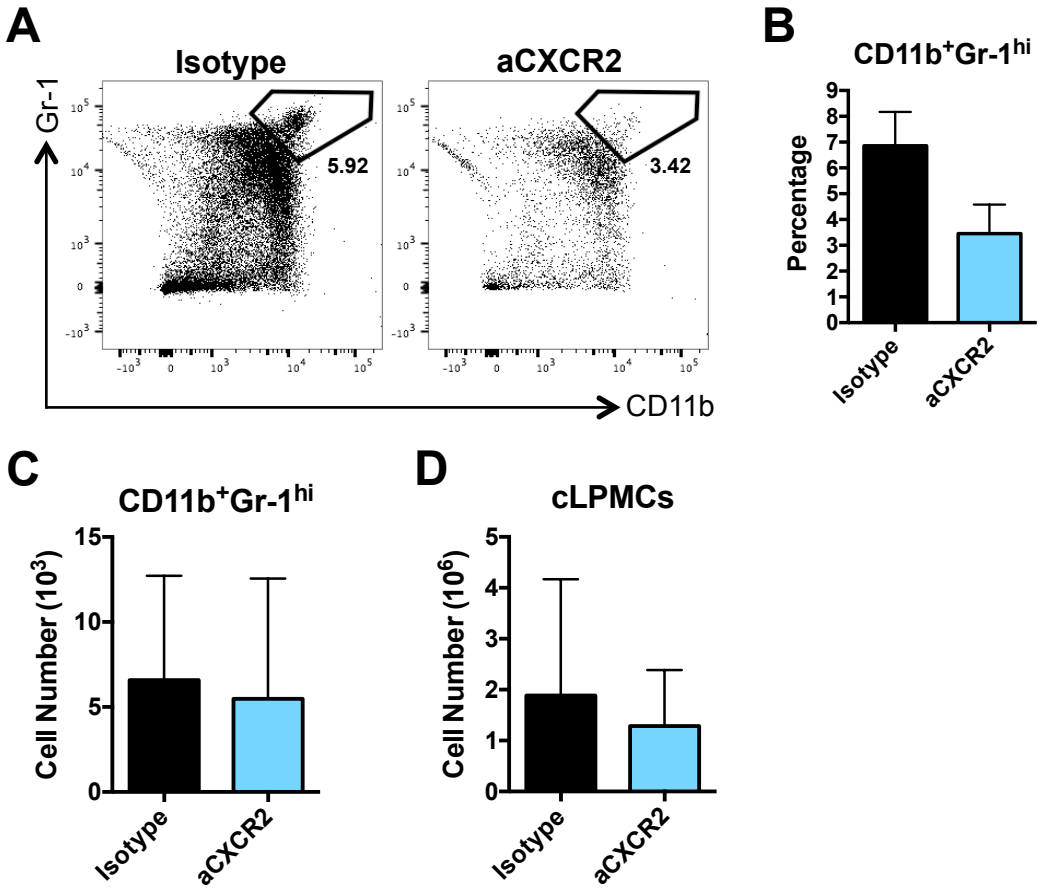

Figure 44: CXCR2 blockade didn't seem to affect neutrophil recruitment to the cLP of TRUC mice. TRUC mice were administered *ip.* with 100µg aCXCR2 or isotype antibody (control) at day 0, 3, 7, 10, 14 and culled at day 15 for downstream analysis. cLPMCs were isolated using Percoll gradient and stained with Abs against surface markers to analyse neutrophil infiltrate by FACS. A. Representative dot plots showing the percentage of neutrophils defined as $CD11b^{+}Gr\text{-}1^{hi}$ cells in the cLP of TRUC mice treated with 100µg of aCXCR2 (n=3) or isotype control Ab (n=2), respectively. Cells gated as single live $CD45^{+}$ cells. Data representative of a single experiment. B. Graph showing the percentage of $CD11b^{+}Gr\text{-}1^{hi}$ cells in the cLP of TRUC mice treated with 100µg of aCXCL1 (n=3) or isotype control Ab (n=2), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). C. Graph showing the absolute number of $CD11b^{+}Gr\text{-}1^{hi}$ cells in the cLP of TRUC mice treated with 100µg of aCXCR2 (n=3) or isotype control Ab (n=2), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). D. Graph showing the absolute number of cLPMCs in TRUC mice treated with 100µg of aCXCR2 (n=3) or isotype control Ab (n=2), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

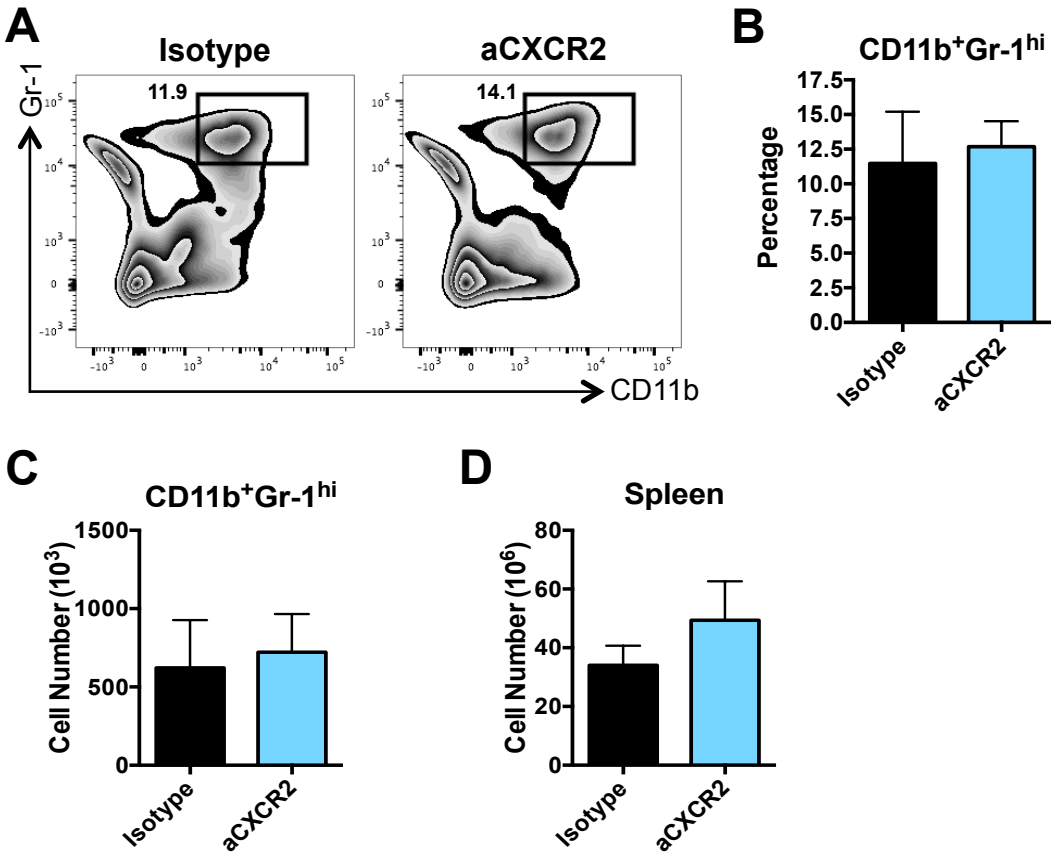

Figure 45: CXCR2 blockade didn't seem to affect neutrophil recruitment to the spleen of TRUC mice. TRUC mice were administered *ip.* with 100µg aCXCR2 or isotype antibody (control) at day 0, 3, 7, 10, 14 and culled at day 15 for downstream analysis. Single cell suspensions were prepared from the spleens of TRUC mice treated with 100µg aCXCR2 (n=4) or isotype control Ab (n=10) and stained with Abs against surface markers to analyse neutrophil infiltrate by FACS. A. Representative dot plots showing the percentage of neutrophils defined as $CD11b^{+}Gr\text{-}1^{hi}$ cells in the spleen of TRUC mice treated with 100µg of aCXCR2 (n=4) or isotype control Ab (n=10), respectively. Cells gated as single live $CD45^{+}$ cells. Data representative of a single experiment. B. Graph showing the percentage of $CD11b^{+}Gr\text{-}1^{hi}$ cells in the spleen of TRUC mice treated with 100µg of aCXCR2 (n=4) or isotype control Ab (n=10), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). C. Graph showing the absolute number of $CD11b^{+}Gr\text{-}1^{hi}$ cells in the spleen of TRUC mice treated with 100µg of aCXCR2 (n=4) or isotype control Ab (n=10), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). D. Graph showing the absolute number of splenocytes in TRUC mice treated with 100µg of aCXCR2 (n=4) or isotype control Ab (n=10), respectively. Data representative of a single experiment. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

5.7 IL-22 seems to induce CXCL5 production in a small intestinal epithelial cell line

The data presented in this chapter collectively support the hypothesis that NCR$^-$ ILC3s and IL-22 are involved in the regulation of neutrophil recruitment to the colon in TRUC disease. This process could be mediated by the known neutrophil chemo-attractants CXCL1 and CXCL5 that are produced by colonic epithelial cells in response to IL-22 stimulation. However, the mechanism of how IL-22 acts on the colonic epithelium to induce the production of the above chemokines is still unknown.

In an attempt to identify the molecules involved in the downstream signalling of the IL-22R, a small intestinal epithelial cell line (Mode K) was obtained. Mode K were grown and expanded in culture, and once confluent they were stimulated for 24h with 10ng/ml of recombinant IL-22 with or without commercially purchased inhibitors for further analysis. Unfortunately, as shown in Figure 46A, IL-22 stimulation failed to induce the expression of *Cxcl5* in Mode K cells.

Similarly, 24h stimulation with IL-22 didn't induce CXCL5 production by Mode K cells Mode K cells (Figure 48B). As such, no conclusions could be drawn by the use of inhibitors that are known to block the IL-22 signaling pathway. Different settings are therefore needed in order to understand how IL-22 acts on the colonic epithelium.

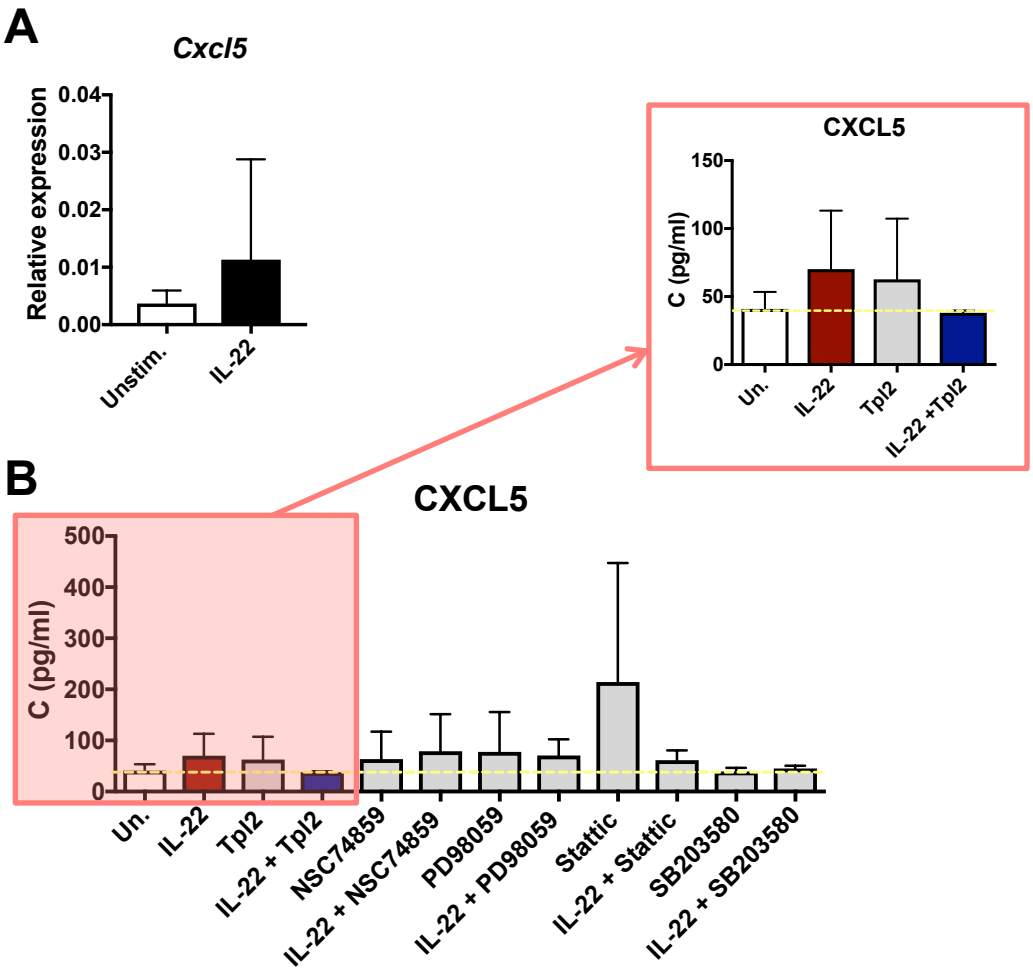

Figure 46: *Cxcl5* expression in Mode K cells. Mode K cells were cultured until confluent then transferred in 6-well plates at a concentration of $2x10^6$cell/well and stimulated for 24h with 10ng/ml recombinant IL-22. mRNA and protein levels of CXCL5 were measured by RT-qPCR and ELISA, respectively. A. *Cxcl5* expression in Mode K cells stimulated for 24h with or without 10ng/ml recombinant IL-22 measured by RT-qPCR. Data representative of two independent experiments with 3 technical replicates. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001). B. CXCL5 production by Mode K cells stimulated for 24h with 10ng/ml recombinant IL-22 in the presence or absence of several IL-22 signaling pathway inhibitors. CXCL5 concentration in the S/Ns was measured by ELISA. Data representative of a single experiment with 3 technical replicates. Statistical analysis was performed using PRISM® version 7 software (Mann-Whitney two-tailed test, *p<0.05, **P<0.005, ***p<0.0001).

5.8 Discussion

In the previous two chapters (3 and 4) of this thesis, it was shown that IL-22 has a key role in TRUC disease, quite possibly through induction of ER stress in colonic epithelial cells. The data shown in this chapter suggest that IL-22 might also regulate neutrophil recruitment to the colonic LP by inducing the production of chemokines CXCL1 and CXCL5 by colonic epithelial cells.

Neutrophil accumulation to the colonic LP is an important aspect of TRUC pathology (Powell, Walker et al. 2012, Ermann, Staton et al. 2014). Transcripts encoding for several CXC and CCL chemokines were increased in the colon of TRUC mice compared to *Rag2*$^{-/-}$ control mice. Similar transcriptional changes were observed in colonic epithelial cells following 24h stimulation with IL-22. These findings were also corroborated by RT-qPCR performed by Dr Anastasia Tsakmaki. The production of CXCL1 and CXCL5, two known neutrophil chemo-attractants, were also significantly increased in the supernatants of colonois treated with IL-22. These transcriptomic data support the hypothesis of IL-22 having an additional effect on the colonic epithelium by inducing chemokine production that subsequently leads to neutrophil accumulation to the gut.

Interestingly, a co-culture experiment of FACS purified NCR^{-} ILC3s isolated from TRUC and TRUC*Il22*$^{-/-}$ mice with murine colonoids revealed that only IL-22 sufficient NCR^{-} ILC3s were able to significantly induce the expression of the chemokine genes *Cxcl1* and *Cxcl5* in colonic epithelial cells, results which were also confirmed by ELISA on the supernatants of these cultures. These findings suggest that NCR^{-} ILC3s may also contribute to TRUC pathology via neutrophil recruitment to the inflamed colon in TRUC mice through IL-22 mediated induction of CXCL1 and CXCL5 production by colonic epithelial cells.

Pro-inflammatory chemokines are highly expressed in CD and UC patients (Reinecker, Loh et al. 1995, Ina, Kusugami et al. 1997, Huang, Eckmann et al. 1996), suggesting their importance in disease pathology. Consistent with a disease-free phenotype TRUC*Il22*$^{-/-}$ mice had significantly lower neutrophil infiltrate in their spleens and colons compared to TRUC mice. Moreover, IL-22 blockade significantly reduced the absolute number of neutrophils in the colonic LP of TRUC mice, while *in vivo* administration of recombinant IL-22 into TRU*Il22*$^{-/-}$ mice led to a

significant increased neutrophil accumulation to the colon of these mice. These findings suggest a key role for IL-22 in the regulation of neutrophil recruitment to the gut, and in particular to the colon, that may comprise another mechanism by which NCR^- ILC3s contribute to TRUC pathology.

Interestingly, in contrast to what was seen in the colon, administration of rIL-22 into TRUC*Il22*$^{-/-}$ mice resulted in a significantly decrease in the percentage of neutrophils found in the spleen of these mice suggesting that there may be alternative mechanisms that regulate neutrophil trafficking to different tissues. Whether this is indeed the case is yet to be determined as this experiment was only performed once due to limited mouse availability, so repeating these experiments is needed in order to confirm/validate these findings.

Surprisingly, *in vivo* CXCL1 blockade didn't affect neutrophil accumulation to the colonic LP of TRUC mice, although the experiment needs to be repeated in order to confirm the data. Similarly, *in vivo* blocking of the chemokine receptor for CXCL1 and CXCL5, CXCR2, had no impact on neutrophil infiltrate in the colonic LP, which appears to challenge the notion of CXCR2 having an important role in neutrophil accumulation and disease outcome (Buanne, Di Carlo et al. 2007). However, these findings can only be characterized as preliminary as they came from a single experiment with very small n numbers.

Finally, in an attempt to scrutinize the signalling pathway by which IL-22 induced CXCL1 and CXCL5 production by colonic epithelial cells, a small intestinal cell line (Mode K cells) was exploited. However, *in vitro* stimulation with IL-22 in the presence or absence of inhibitors that block different components of the IL-22 signaling pathway, failed to induce CXCL1 and CXCL5 production by colonic epithelial cells which suggests that maybe IL-22 has different effects on small intestinal epithelial cells than those observed in the colonic epithelium of TRUC mice, and hence alternative experiments are necessary in order to understand the mechanisms by which IL-22 impacts on colonic epithelial cells contributing to TRUC pathology.

In general, neutrophil accumulation to the gut is thought to contribute in the promotion of intestinal inflammation (Zimmerman, Vongsa et al. 2008). Taken all together, the data presented in this chapter suggest that IL-22 may regulate neutrophil

recruitment to the gut by inducing the production of CXCL1 and CXCL5 by colonic epithelial cells, which in turn may constitute an additional pro-inflammatory program used by NCR^- ILC3s to drive disease in TRUC mice.

Chapter 6

Results: IL-22 regulated/TRUC relevant transcripts are increased in patients with active UC

In the first results chapter (chapter 3), it was shown that TRUC disease is characterized by an IL-22 responsive transcriptional signature, and most importantly that IL-22 drives intestinal inflammation in the colon of TRUC mice. Moving on to investigate how exactly IL-22 mediates inflammation in TRUC disease, in chapter 4 it was shown that IL-22 produced by NCR^- ILC3s induced ER stress in colonic epithelial cells, and that that induction was significantly reduced in the presence of regulatory T cells (which are normally absent in TRUC mice due to *Rag2* deficiency). Moreover, *in vivo* blocking of ER stress attenuated inflammation in TRUC mice. An additional effect of IL-22 on the colonic epithelium was then described on chapter 5, where it was shown that IL-22 regulates neutrophil accumulation to the colonic LP by inducing the production of the chemokines CXCL1 and CXCL5 by colonic epithelial cells. Taken all together, these data point to IL-22 being a key regulator of colonic inflammation in TRUC disease.

The TRUC mouse is a great model of chronic colitis that resembles many aspects of human UC (Garrett, Lord et al. 2007). To investigate whether the findings described so far in this thesis bare any relevance to human disease, and in particular to UC, reposited datasets with availability on whole genome expression profiles of gut tissue from UC patients and healthy individuals were interrogated. Among several datasets publicly available, the largest one published by Vanhove et al (Vanhove, Peeters et al. 2015) used RNA extracted from colonic biopsies of patients with active UC (n=74), patients with inactive disease (n=23) and healthy individuals (n=11) was selected for further analysis. Raw data were obtained from the Gene Expression Omnibus repository (accession number GSE59071) and were analysed using Partek® Genomics Suite software and GraphPad PRISM® version 7 software.

6.1 Upregulated genes in TRUC disease are also upregulated in patients with active UC

As shown in Figure 47, some of the most upregulated genes found in TRUC mice (compared to healthy *Rag2*$^{-/-}$ controls) like *Cxcl1*, *Duoxa2*, *S100a8* were also significantly increased in the mucosa of patients with active disease compared to healthy individuals. Notably, among them were also IL-22 responsive genes (highlighted with pattern in Figure 47) as defined by gene expression analysis (Mouse Gene 2.0 ST array, Affymetrix) in IL-22 treated colonoids compared to untreated ones, suggesting that IL-22 may also be relevant in human disease.

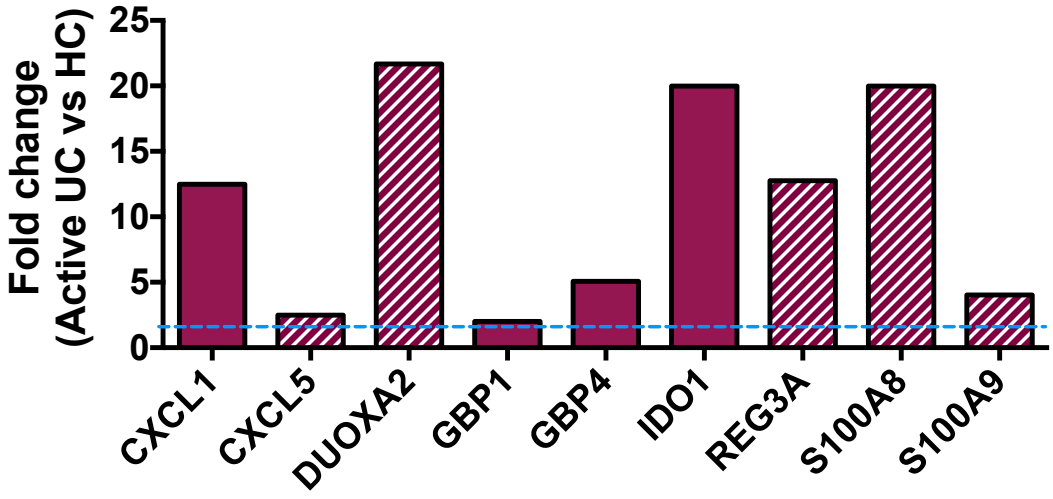

Figure 47: Transcripts of TRUC upregulated genes are increased in patients with active UC. Graph showing fold change of upregulated genes in colonic biopsies from patients with active UC vs. healthy controls. Raw data were obtained from the Gene Expression Omnibus (GEO) repository (Accession number GSE59071) (Vanhove, Peeters et al. 2015). Analysis was performed using Partek® and GraphPad PRISM® version 7 software.

6.2 *IL22* and other IBD relevant transcripts are increased in patients with active UC

Supporting this hypothesis, the expression of *IL22* was significantly upregulated in patients with active disease compared to healthy individuals (p=0.0017) or patients with quiescent UC (p< 0.0001) (Figure 48). As expected, transcripts of known IBD relevant genes were also significantly increased in patients with active disease compared to healthy individuals or patients with quiescent UC as shown in Figures 48 and 49 (*IL17A*: p< 0.0001, *IFNG*: p< 0.0001, *TNFA*: p< 0.0001, *TNFSF15*: p< 0.0001 respectively).

Interestingly, as shown in Figures 50 and 51, *IL22* expression in patients with active disease was significantly positively correlated with the expression of *IL17A*, *IFNG*, *TNFA* and *TNFSF15* (p= 0.0031, p<0.0001, p=0.0048, and p= 0.0413 respectively) in the same individuals.

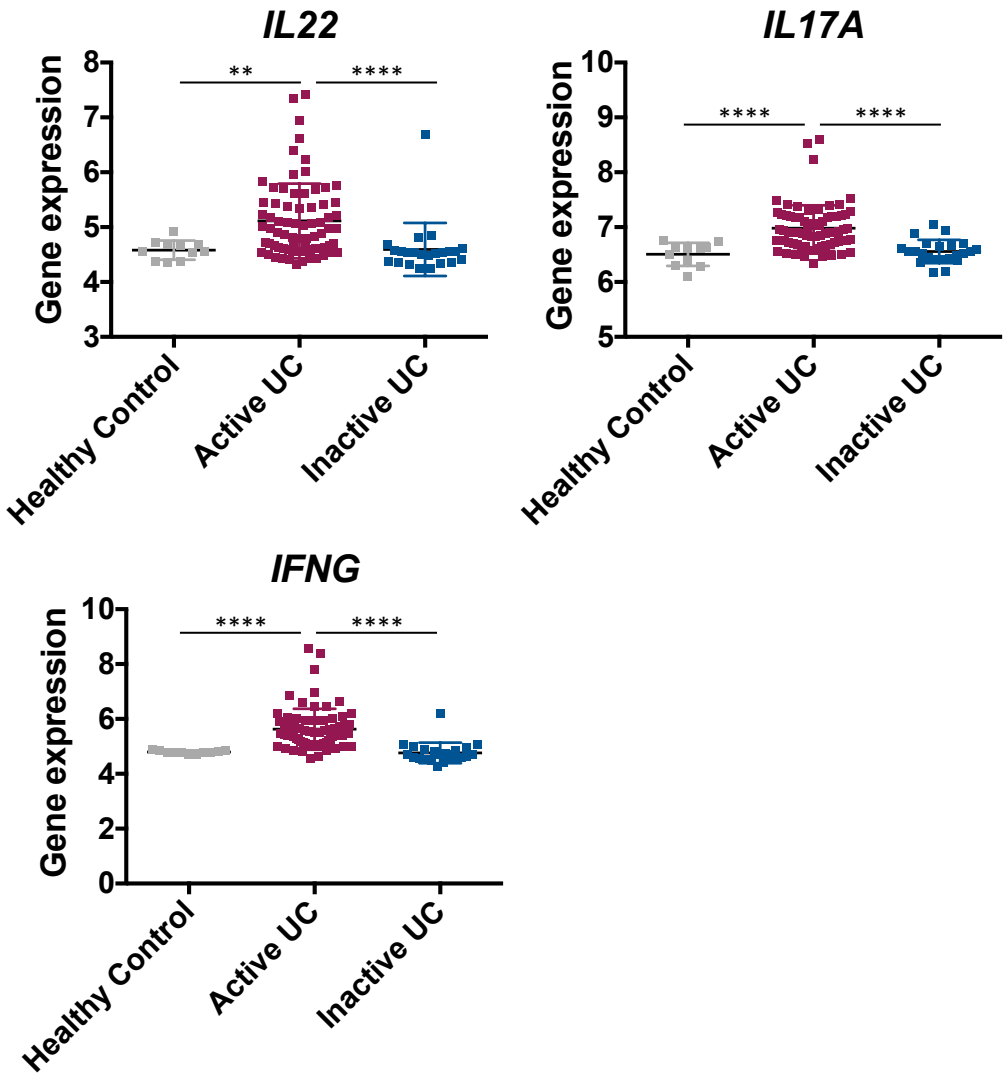

Figure 48: IL-22 and other TRUC relevant cytokine expression in human UC. Graphs showing expression levels of IL22, IL17A and IFNG respectively in the colon of patients with active UC vs. patients with quiescent UC and healthy controls. Each square represents one patient. Lines depict means with SD. Raw data were obtained from the Gene Expression Omnibus (GEO) repository (Accession number GSE59071) (Vanhove, Peeters et al. 2015). Analysis was performed using Partek® and GraphPad PRISM® version 7 software.

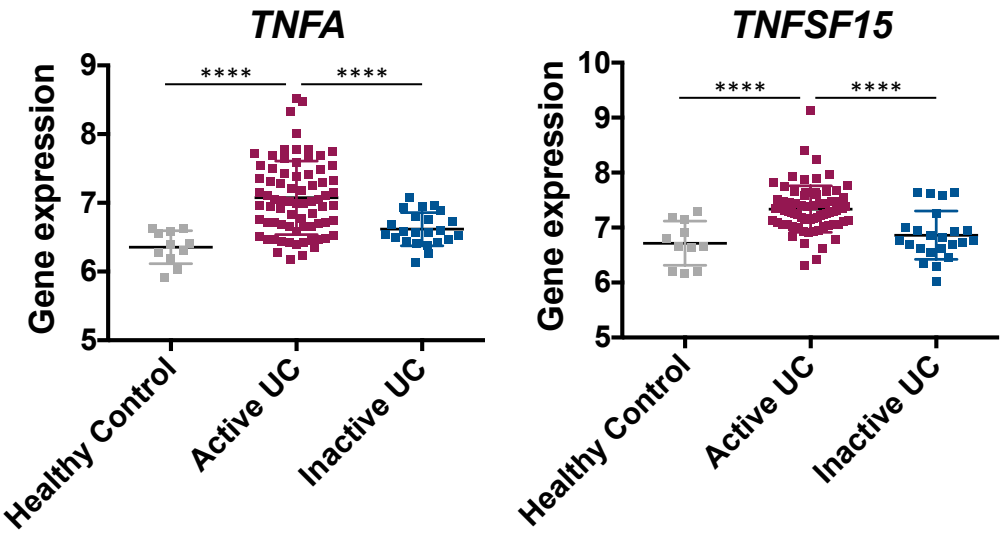

Figure 49: *TNFA* and *TNFSF15* expression in human UC. Graphs showing expression levels of TNFA and TNFSF15 respectively in the colon of patients with active UC vs. patients with quiescent UC and healthy controls. Each square represents one patient. Lines depict means with SD. Raw data were obtained from the Gene Expression Omnibus (GEO) repository (Accession number GSE59071) (Vanhove, Peeters et al. 2015). Analysis was performed using Partek® and GraphPad PRISM® version 7 software.

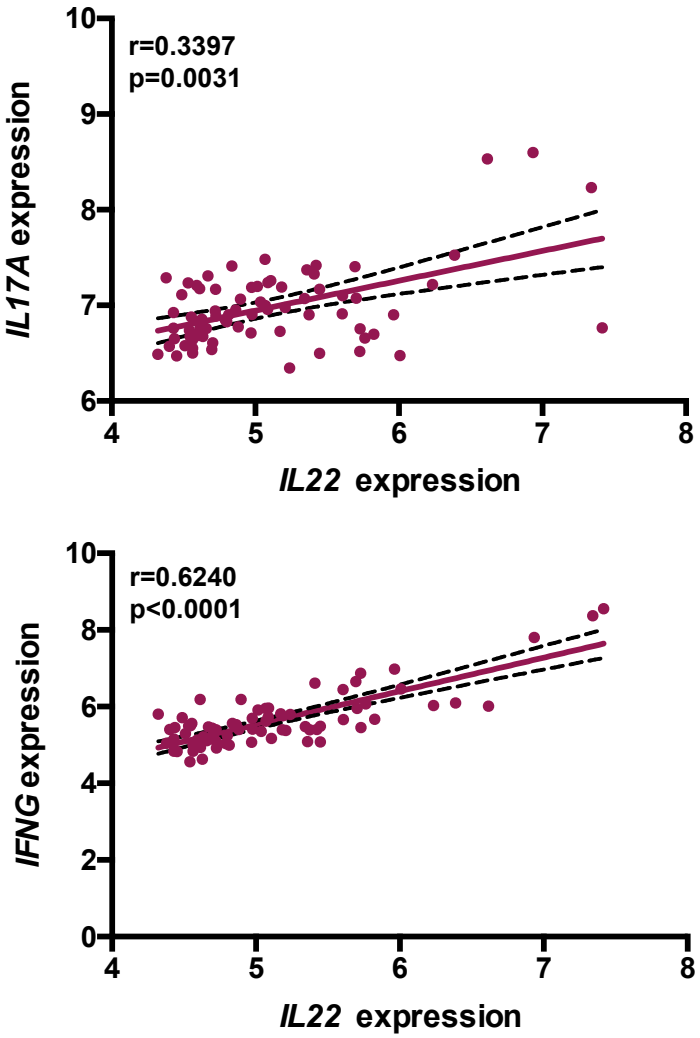

Figure 50: *IL22* expression is significantly positively correlated with the expression of *IL17A* and *IFNG* in patients with active UC. Graphs showing how the expression levels of IL22 correlate with those of IL17A and IFNG respectively in the colon of patients with active UC (best-fit line with 95% confidence band). Each square represents a single patient. Raw data were obtained from the Gene Expression Omnibus (GEO) repository (Accession number GSE59071) (Vanhove, Peeters et al. 2015). Analysis was performed using Partek® and GraphPad PRISM® version 7 software.

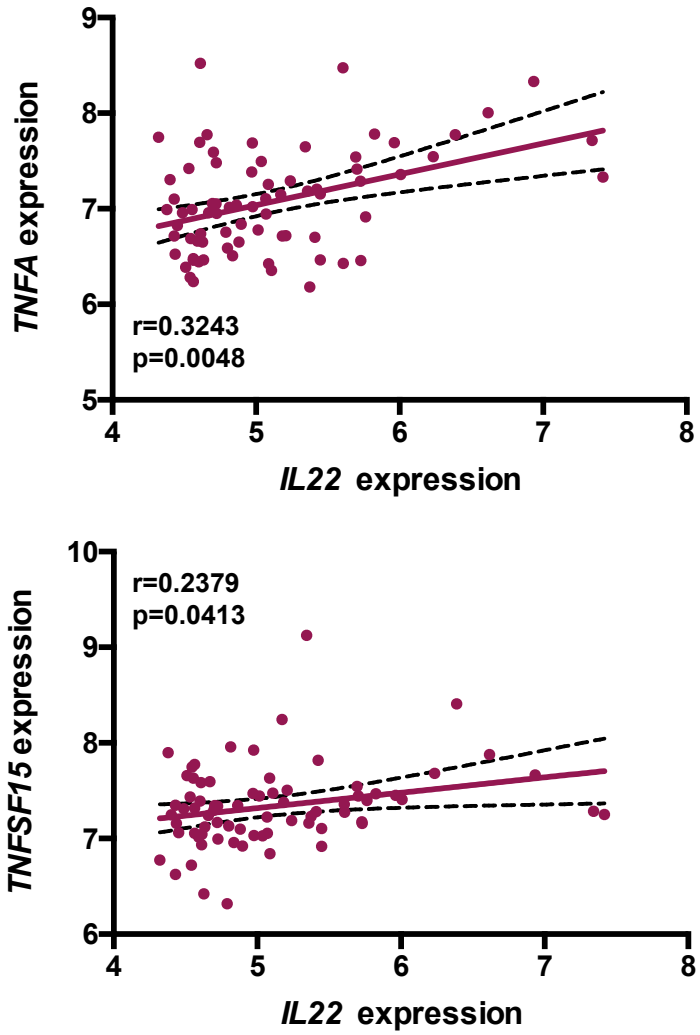

Figure 51: *IL22* expression is significantly positively correlated with the expression of *TNFA* and *TNFSF15* in patients with active UC. Graphs showing how the expression levels of IL22 correlate with those of TNFA and TNFSF15 respectively in the colon of patients with active UC (best-fit line with 95% confidence band). Each square represents a single patient. Raw data were obtained from the Gene Expression Omnibus (GEO) repository (Accession number GSE59071) (Vanhove, Peeters et al. 2015). Analysis was performed using Partek® and GraphPad PRISM® version 7 software.

6.3 *ER stress* transcripts are increased in patients with active UC

Similarly to TRUC disease, transcripts of known ER stress genes such as *XBP1*, *ATF6* and *ERN1* were found to be significantly increased in patients with active disease compared to healthy individuals or patients with quiescent UC (Figure 52).

Moreover, as shown in Figure 53, *IL22* expression was significantly positively correlated with the expression of the ER stress genes *ATF6* and *ERN1* but not with that of *XBP1* in patients with active disease, suggesting that the IL-22 actions found in our preclinical models may also be relevant in human UC.

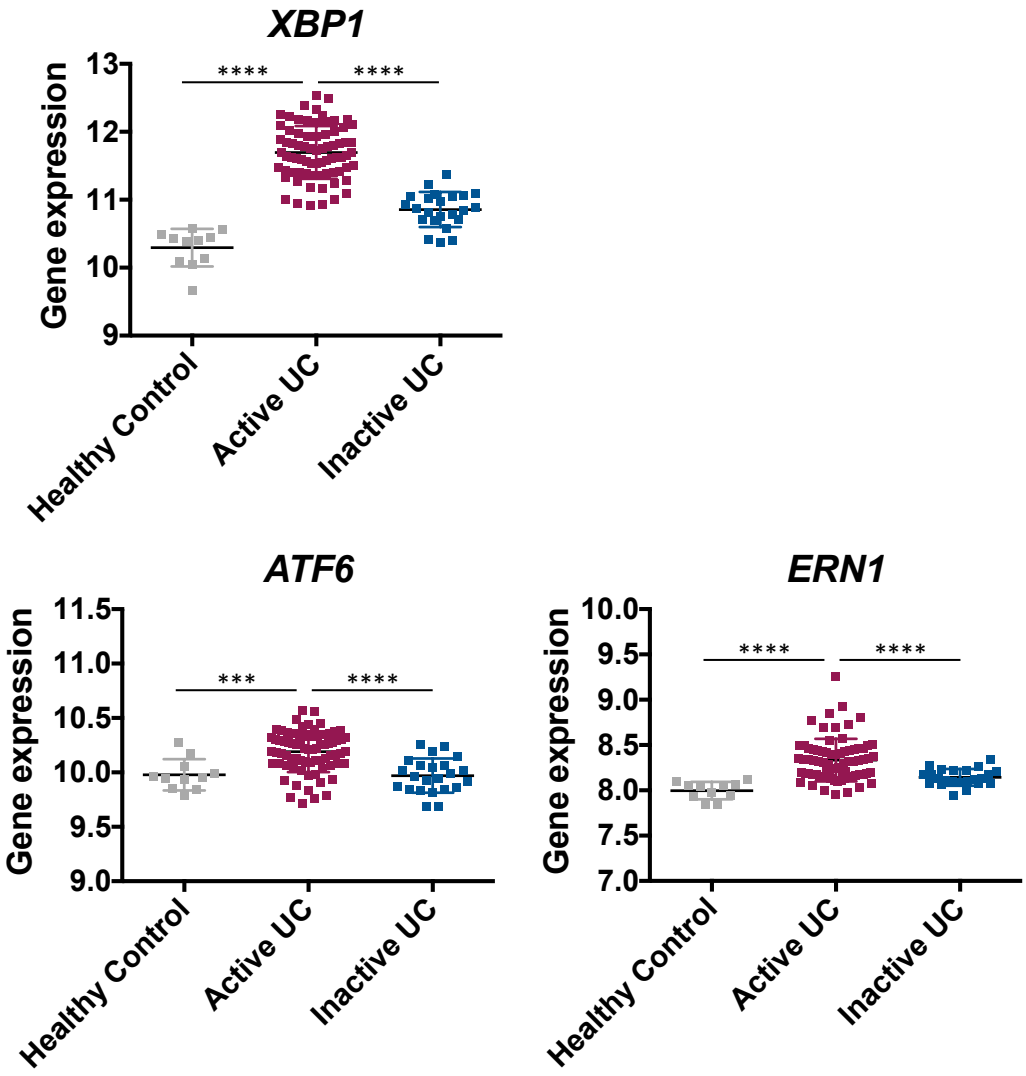

Figure 52: ER stress genes are upregulated in patients with active UC. Graphs showing expression levels of XBP1, ATF6 and ERN1 respectively in the colon of patients with active UC vs. patients with quiescent UC and healthy controls. Each square represents one patient. Lines depict means with SD. Raw data were obtained from the Gene Expression Omnibus (GEO) repository (Accession number GSE59071) (Vanhove, Peeters et al. 2015). Analysis was performed using Partek® and GraphPad PRISM® version 7 software.

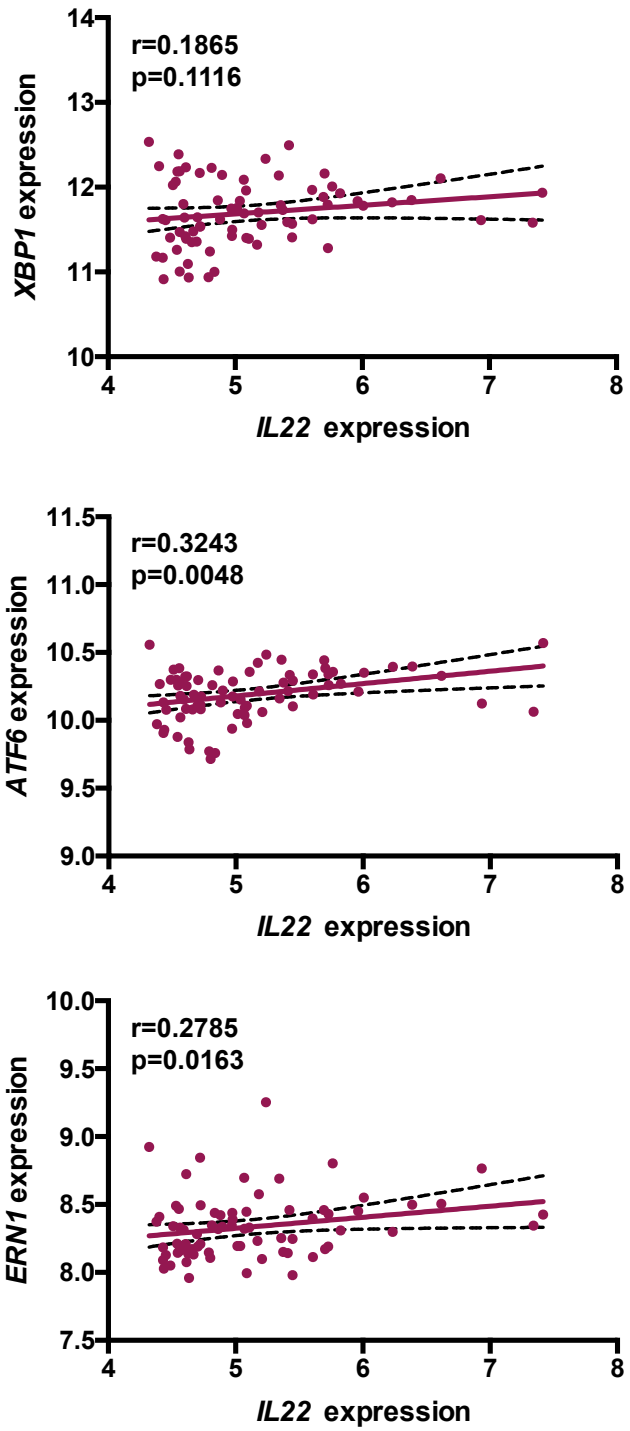

Figure 53: *IL22* expression is significantly positively correlated with the expression of *ATF6* and *ERN1* in patients with active UC. Graphs showing how the expression levels of IL22 correlate with those of ATF6 and ERN1 respectively in the colon of patients with active UC (best-fit line with 95% confidence band). Each square represents a single patient. Raw data were obtained from the Gene Expression Omnibus (GEO) repository (Accession number GSE59071) (Vanhove, Peeters et al. 2015). Analysis was performed using Partek® and GraphPad PRISM® version 7 software.

6.4 Gene expression of *CXCL1* and *CXCL5* is increased in patients with active UC

Supporting this narrative, *CXCL1* and *CXCL5* transcripts were also significantly upregulated in patients with active disease compared to healthy individuals or patients with quiescent UC (Figure 54). More importantly, their expression was positively correlated with *IL22* expression in patients with active disease as shown in Figure 55.

Taking all together, these data support the notion of IL-22 being implicated in UC pathogenesis, possibly by inducing ER stress in the colonic epithelium and/or driving neutrophil recruitment through CXCL1 and CXCL5 production by epithelial cells.

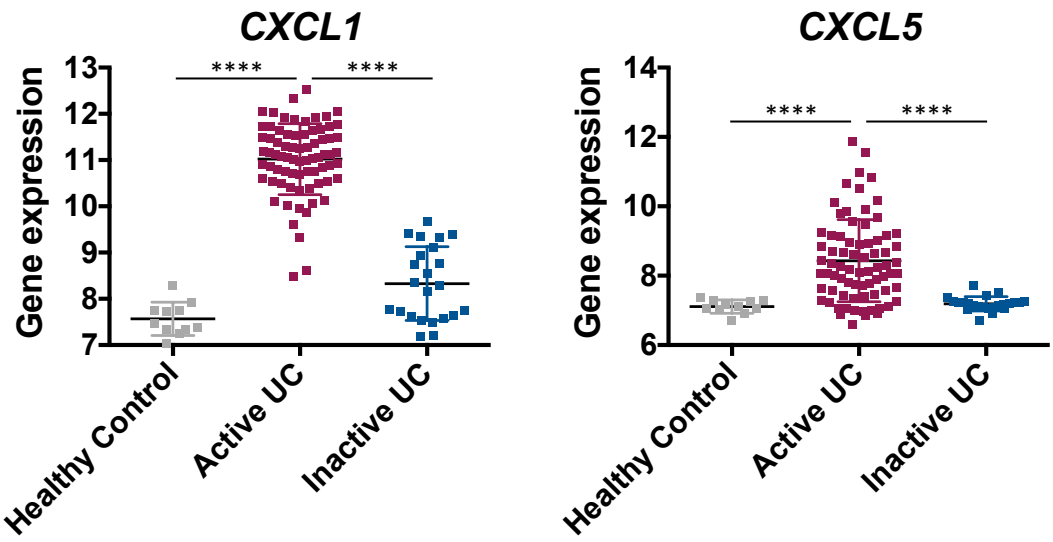

Figure 54: *CXCL1* and *CXCL5* transcripts are increased in patients with active UC. Graphs showing expression levels of CXCL1and CXCL5 respectively in the colon of patients with active UC vs. patients with quiescent UC and healthy controls. Each square represents one patient. Lines depict means with SD. Raw data were obtained from the Gene Expression Omnibus (GEO) repository (Accession number GSE59071) (Vanhove, Peeters et al. 2015). Analysis was performed using Partek® and GraphPad PRISM® version 7 software.

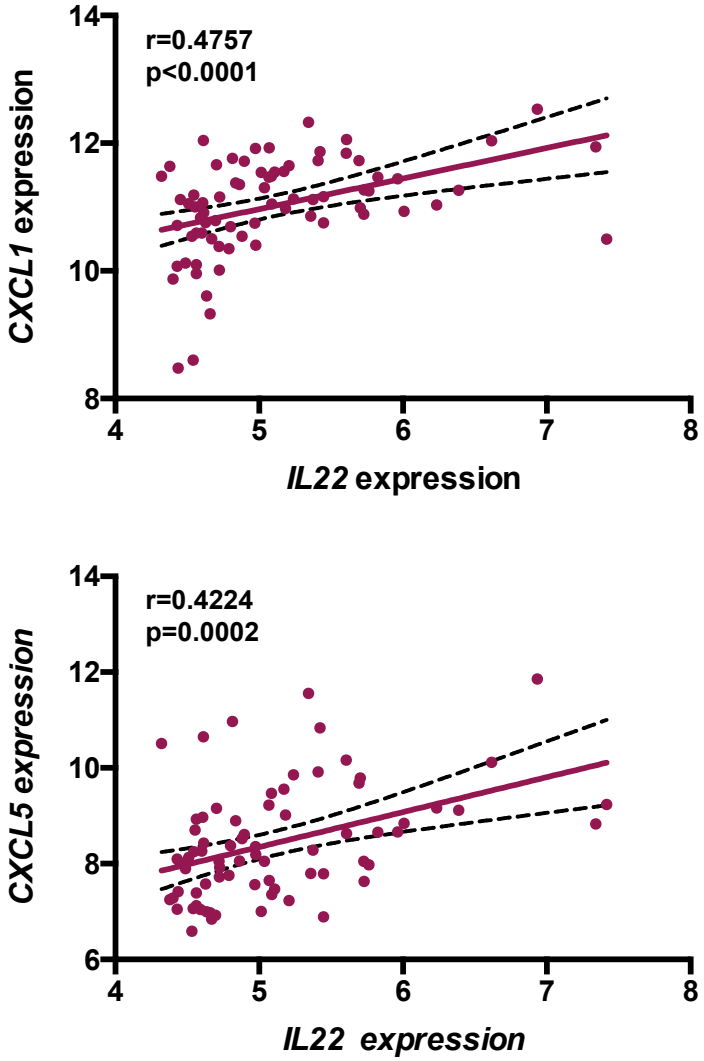

Figure 55: *IL22* expression is significantly positively correlated with the expression of *CXCL1* and *CXCL5* in patients with active UC. Graphs showing how the expression levels of IL22 correlate with those of CXCL1 and CXCL5 respectively in the colon of patients with active UC (best-fit line with 95% confidence band). Each square represents a single patient. Raw data were obtained from the Gene Expression Omnibus (GEO) repository (Accession number GSE59071) (Vanhove, Peeters et al. 2015). Analysis was performed using Partek® and GraphPad PRISM® version 7 software.

6.5 Discussion

In the previous chapters of this thesis, it was shown that TRUC disease is characterized by an IL-22 responsive transcriptional signature, and that IL-22 drived intestinal inflammation in the colon of TRUC mice by inducing ER stress in colonic epithelial cells. In addition, it was shown that IL-22 regulates neutrophil recruitment to the colonic LP by inducing chemokine production, and in particular the expression of CXCL1 and CXCL5 by colonic epithelial cells. In this chapter, it was shown that the pro-inflammatory pathways imprinted by NCR^- ILC3s through IL-22 production in TRUC disease that were described so far may be also relevant in human disease.

The TRUC mouse model of experimental colitis resembles many aspects of human UC (Garrett, Lord et al. 2007). Whole transcriptome analysis on colonic biopsies from patients with active or inactive UC and healthy controls revealed that the human orthologues of several transcripts, which were found highly upregulated in TRUC disease, were also significantly increased in patients with active UC compared to those with inactive disease or to healthy individuals. Within this gene set were also know IL-22 responsive genes suggesting that IL-22 may also be important in the human disease.

Similarly to other cytokines that are known to be important in IBD pathogenesis such as IFNγ, IL-17A, TNFα and TL1A the transcripts of which were found to be significantly increased in patients with active UC compared to inactive disease or healthy controls, *IL22* expression was also significantly higher in patients with active disease. Interestingly, *IL22* expression in patients with active disease was significantly positively correlated with the expression of *IFNG*, *IL17A*, *TNFA* and *TNFSF15* supporting further the possibility of IL-22 having a functional role in human UC.

Several independent studies have associated ER stress with IBD susceptibility/pathogenesis (Kaser, Lee et al. 2008, Shkoda, Ruiz et al. 2007, Deuring, de Haar et al. 2012). Consistent with our pre-clinical findings, transcripts encoding for known ER stress genes such as *ATF6*, *ERN1* and *XBP1* were significantly increased in patients with active UC compared to those with quiescent disease and healthy individuals. Moreover, the expression of these genes was

positively correlated with *IL22* expression in patients with active UC, suggesting that IL-22 may also induce ER stress in human IBD.

In accordance with studies shown increased chemokine production in IBD patients (Reinecker, Loh et al. 1995, Ina, Kusugami et al. 1997, Huang, Eckmann et al. 1996), *CXCL1* and *CXCL5* transcripts were significantly upregulated in patients with active disease compared to those with inactive UC and healthy controls, and their expression was positively correlated with *IL22* expression in patients with active UC, supporting the possibility of IL-22 regulating neutrophil recruitment to the gut by inducing chemokine production by colonic epithelial cells in human UC.

Although these preliminary findings suggest that IL-22 pro-inflammatory actions in our pre-clinical model of IBD described in this thesis may be also relevant in human disease, some more systematic approaches would have been of additional value. GSEA would reveal in an unbiased way whether the most upregulated genes found in TRUC disease were enriched in patients with active disease, and similarly whether the IL-22 responsive genes found in our colonoid system were also enriched in human disease. Moreover, pathways analysis would be beneficial to identify specific pathways associated with human UC, and in particular all the ER stress components, the transcription of which may be affected in UC. Similarly, as several genetic loci have been associated with both CD and UC susceptibility (Jostins, Ripke et al. 2012), the same analysis could be extended to patients with active and inactive CD, to see whether IL-22 may be relevant in human CD.

Finally, these findings are only based on transcriptomic analysis and although they may suggest that the pathways described in the previous chapters of this thesis could also be relevant in human disease, they should solely be treated as an indication of such hypothesis, whilst additional human work is necessary to investigate this further.

Chapter 7

Conclusions

7.1 New insights into the pathology of TRUC disease

The data presented in this thesis shed more light into the immunopathology of TRUC disease. As originally described by *Garrett et al* T-bet deficiency in the absence of an adaptive immune system leads to chronic colitis that resembles many aspects of human UC (Garrett, Lord et al. 2007). Previous work in the lab by *Powell et al* showed that in TRUC mice, colonic $CD103^{-}CD11b^{+}$ DCs produce excessively TNFα and IL-23, which leads to the activation of ILC3s that drive disease through IL-17A production (Powell, Walker et al. 2012). The data presented in this thesis suggest that NCR^{-} ILC3s, the most abundant ILC3 subset in the colonic LP, may drive disease through induction of ER stress in colonic epithelial cells and regulation of neutrophil recruitment through induction of chemokine production by colonic epithelial cells. It was also shown that NCR^{-} ILC3s mediate these pro-inflammatory functions through the production of IL-22. Finally, it was shown that the pro-inflammatory pathways found in our pre-clinical model of IBD might also be relevant in human disease. Taken all together, the data described in this thesis expand our current understanding of TRUC disease and propose new mechanisms by which NCR^{-} ILC3s may drive inflammation in chronic colitis that could be further explored in order to define new therapeutic strategies to treat IBD.

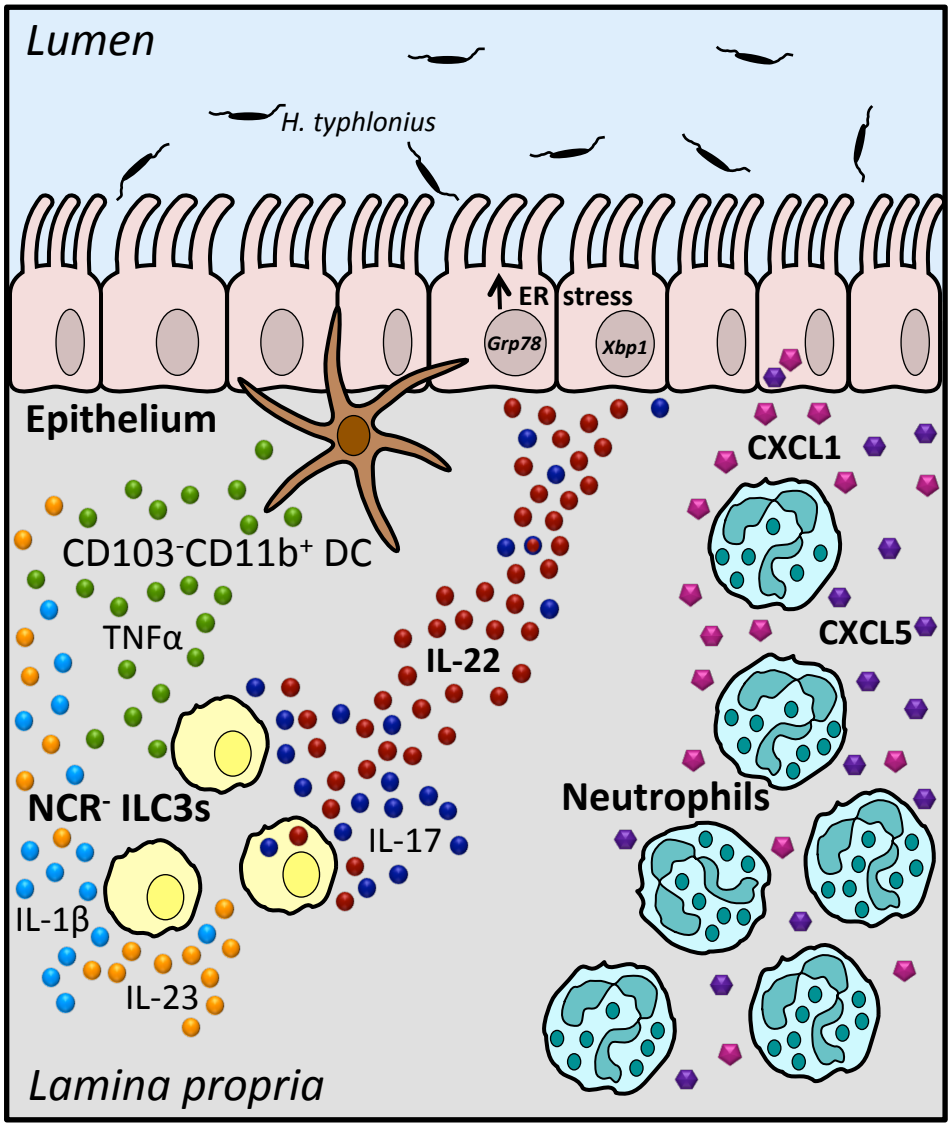

Figure 56: Updated model of TRUC pathology. In the colonic lamina propria of TRUC mice, where DCs sample antigens from the lumen, CD103$^-$CD11b$^+$ DCs produce excessively TNFα and IL-23 that results in the activation of NCR$^-$ ILC3s. Upon activation, NCR$^-$ ILC3s produce high amounts of IL-17A and IL-22. Here, it was shown that IL-22 induced ER stress in the colonic epithelial cells and regulated neutrophil recruitment to the colon by inducing chemokine production by colonic epithelial cells promoting intestinal inflammation.

7.2 New implications of NCR$^-$ ILC3s in IBD

The data presented in this thesis, described for the first time new pro-inflammatory functions for NCR$^-$ ILC3s in TRUC disease. In contrast to NCR$^+$ ILC3s, NCR$^-$ ILC3s have not been extensively studied in the IBD, and so this thesis aimed to define their role in chronic colitis. NCR$^-$ ILC3s were found to be the most abundant ILC3 subset in the colonic LP in healthy steady and during intestinal inflammation. It was shown that they have a crucial role in chronic colitis mediated by production of IL-22 that subsequently led to ER stress induction in colonic epithelial cells and neutrophil recruitment to the colon. In the gut, it's suggested that IL-22 is beneficial, as it promotes host's defence against commensal bacterial and foreign pathogens (Sonnenberg, Monticelli et al. 2012), as well as tissue regeneration (Lindemans, Calafiore et al. 2015), whereas the data presented in this thesis challenge that notion, additional work is necessary to define whether these findings could also be relevant in other murine models of experimental colitis where immuno-competent mice are used, and ultimately to whether or not are relevant in human disease.

TRUC*Il22*$^{-/-}$ mice are protected from disease, while *in vivo* blockade of IL-22 attenuated disease and reduced colonic ER stress in TRUC mice. Several independent studies have implicated ER stress with IBD susceptibility and pathogenesis (Kaser, Lee et al. 2008, Shkoda, Ruiz et al. 2007, Deuring, de Haar et al. 2012). For the first time, this physiological cellular process is linked with NCR$^-$ ILC3s, suggesting a pro-inflammatory role for these cells in chronic inflammation. Although during acute epithelial injury (Zenewicz, Yancopoulos et al. 2008, Sugimoto, Ogawa et al. 2008) or upon intestinal infections, where homeostasis is quickly restored, IL-22 has beneficial properties (Zheng, Valdez et al. 2008, Monticelli, Sonnenberg et al. 2011), it is possible that during chronic inflammation IL-22 mediates more pathological functions. In psoriasis, IL-22 is thought to be pathogenic (Wolk, Witte et al. 2009, Wolk, Haugen et al. 2009), which could also support that hypothesis. IL-22 was also shown to regulate neutrophil accumulation to the colon possibly by inducing the production of CXCL1 and CXCL5 by colonic epithelial cells. Pro-inflammatory chemokines are highly expressed in IBD patients (Reinecker, Loh et al. 1995, Ina, Kusugami et al. 1997, Huang, Eckmann et al. 1996), while neutrophil infiltration in the gut is thought to be associated with disease

pathology (Zimmerman, Vongsa et al. 2008), highlighting the importance of this pathway in IBD pathogenesis.

The pro-inflammatory pathways described in this thesis directly linking NCR$^-$ ILC3s with TRUC pathology, also appeared to be relevant in human disease. Transcripts encoding IL-22, ER stress genes and the chemokines CXCL1 and CXCL5 were significantly increased in patients with active UC, suggesting that the pro-inflammatory programs triggered by IL-22 may also be important in human disease. Additional human studies are necessary to investigate this hypothesis and explore the pathways involved.

Further exploration of these pathways may lead to the development of new therapeutic strategies to treat IBD. The data presented here support the hypothesis that blocking antibodies against IL-22 could be beneficial to patients with UC. With several studies describing a detrimental role for IL-22 in different cancer, and in particular in colorectal cancer (Huang, Cao et al. 2015, Koltsova, Grivennikov 2014, Kryczek, Lin et al. 2014), which in certain cases can be associated with IBD, these findings highlight even more the role of this effector cytokine in intestinal inflammation and enhance the need for additional studies.

In summary, the data presented in this thesis provide new evidence on the role of NCR$^-$ ILC3s and their effector cytokine IL-22 in chronic colitis, which could be relevant in human disease, and offer new directions that can be explored in order to create novel therapeutic strategies to treat IBD.

7.3 Future directions

Future work will focus on identifying the exact mechanisms and signaling pathway(s) by which IL-22 may regulate ER stress, as well as neutrophil recruitment to the gut in TRUC disease. It is also important to see whether these interesting findings in our preclinical model of IBD can be translated to human disease. An additional route that could also be explored is the link between chronic inflammation and colorectal cancer, as IL-22 is thought to be a major player in this context.

Finally, it would be interesting to investigate the role of ILC2s in chronic inflammation, as they seem to be the most abundant ILC subset in the murine colon, which could provide new insights into both ILC biology and IBD pathogenesis.

Chapter 8

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